REMARKS

Entry of he foregoing and still further reexamination and reconsideration of the subject application, as amended, pursuant to and consistent with 37 C.F.R. §1.116, are respectfully requested in light of the following remarks.

Claims 6, 12-26, 31 and 37-51 remain in the application. Claims 6 and 31 are proposed to be amended hereinabove by changing the expression "slowing the progression of diabetic retinopathy" to "slowing the progression toward diabetic retinopathy", to be consistent with the fact that the diabetic being treated is not already suffering from diabetic retinopathy.

The courtesy of the interview granted by the Examiner to the applicant/ inventor, Dr. Stefansson, and to his undersigned representative on April 20, 2005, is gratefully acknowledged. The 35 U.S.C. §§112 and 103 rejections were discussed in detail and it was agreed that applicant's arguments would be submitted in response to the outstanding Official Action and that these remarks would be considered favorably by the Examiner. Those arguments are presented below.

Submitted herewith is a Declaration Pursuant to 37 C.F.R. §1.132 of the inventor, Dr. Stefansson. Dr. Stefansson is a noted authority in ophthalmology and has set forth in his declaration what he knows and/or believes to be true regarding the issues raised by the Examiner in the outstanding Official Action. Where appropriate, Dr. Stefansson has cited literature supporting his positions and copies of the documents cited in the declaration are provided therewith.

Claims 6, 12-26, 31 and 37-51 have been rejected under 35 U.S.C. §112, first paragraph. The specification is not considered enabling for all carbonic anhydrase

inhibitors being used to slow the progression of diabetic retinopathy in a diabetic not suffering from diabetic retinopathy.

However, as pointed out by Dr. Stefansson in his declaration, the systemic sulfonamide drugs that have been used clinically as antiglaucoma agents such as acetazolamide, metazolamide, ethoxyzolamide, dechlorophenamide, dorzolamide, and brinzolamide have in common a basic molecular structure (see *Supuran et al, Carbonic anhydrase inhibitors, Medicinal Research Reviews 23, 146-189, 2003,* see enclosed copy, page 153). These drug molecules inhibit carbonic anhydrase through the same basic molecular structure. Small differences in other parts of the molecules determine pharmacokinetic properties, such as affinity, water solubility and other physical characteristics. The key issue is that the basic molecular structure and the basic function (carbonic anhydrase inhibition) of all these molecules is the same. See also paragraphs [0022] through [0024] of the present application, especially paragraph [0024].

As further noted in his declaration, in studies conducted by Dr. Stefansson and his co-workers of the physiological functions of these molecules, in particular their effect on oxygenation of the retina and optic nerve and vessel diameters, they have tested acetazolamide, dorzolamide, ethoxyzolamide and methoxyzolamide and found their pharmacological functions as they relate to vasodilatation and increased oxygen tension to be the same with slight differences in their potency (*Stefansson E, Pedersen DB, Jensen PK, la Cour M, Kiilgaard JF, Bang K, Eysteinsson T., Optic nerve oxygenation; Prog Retin Eye Res. 2005 May;24(3):307-32.; Bach Pedersen D, Koch Jensen P, la Cour M, Kiilgaard JF, Eysteinsson T, Bang K, Wiencke AK, Stefansson E, Carbonic anhydrase inhibition increases retinal oxygen tension and*

dilates retinal vessels. Graefes Arch Clin Exp Ophthalmol. 2005 Feb;243(2):163-8 and *Stefansson E, Jensen PK, Eysteinsson T, Bang K, Kiilgaard JF, Dollerup J, Scherfig E, la Cour M. , Optic nerve oxygen tension in pigs and the effect of carbonic anhydrase inhibitors, Invest Ophthalmol Vis Sci. 1999 Oct;40(11):2756-61.)* Copies of these references are enclosed.

As Dr. Stefansson explains, the rationale for the effect of the carbonic anhydrase inhibitors is based on their carbonic anhydrase inhibition and in particular their effect on retinal vessel diameter and oxygenation, which he and his co-workers have found to be essentially the same for the various carbonic anhydrase inhibitors mentioned above. It is therefore quite reasonable to expect the clinical effect of all of them to be in line with the pharmacological effect and therefore expect all of them to be useful in preventing the progression to diabetic retinopathy as dorzolamide is shown to be in the clinical study described in the present application.

On page 3 of the Official Action, the Examiner states: "The prior art does not recognize that all carbonic anhydrase inhibitors have the same function. Applicant on page 5 of the specification admits that not all carbonic anhydrase inhibitors have the same therapeutic activity. Applicant admits that some of carbonic anhydrase inhibitors have been used as diuretics for treatment of congestive heart failure or in the treatment of allergies". As noted by Dr. Stefansson, this statement includes a misunderstanding: The class of molecules called carbonic anhydrase inhibitors are indeed classified as such because they have the same function. This common function is the inhibition of the enzyme carbonic anhydrase, including its various isoenzymes. A molecule that does not have this function would not be classified as a carbonic anhydrase inhibitor. What is true, however, is that various molecules in

this class of drug molecules differ in their affinity for the carbonic anhydrase isoenzymes, and some of their physical properties such as water solubility etc.

Dr. Stefansson further notes that the enzyme carbonic anhydrase is ubiquitous in the body and therefore carbonic anhydrase inhibitors can be used for a number of disease conditions such as glaucoma in the eye, and for diureses in the kidney. In both cases, the mechanism of action is through the inhibition of carbonic anhydrase. For example, when acetazolamide is used (rarely) as a diuretic it will also influence the intraocular pressure, and when it is used as a glaucoma drug it will also induce some diureses as a side effect. The desired therapeutic activity may differ with the other therapeutic activities listed as side effects in each case but the actual pharmacologic activity of the drug would be the same. One exception to this, however, is when carbonic anhydrase inhibitors as applied topically to the eye where the majority of the absorption and therapeutic effect will take place in the eye and there will be only a small effect on kidneys and other parts of the body.

Statement 4 on page 3 of the Official Action is not true. Dr. Stefansson points out that the predictability of the pharmaceutical and chemical art is high in the field of carbonic anhydrase inhibitors. The essential structure of sulfonamide carbonic anhydrase inhibitors is well known and well characterized. The difference between the different molecules in the class only affects the pharmacokinetic properties and distribution and not the basic pharmacological action. See the Supuran et al. reference cited hereinabove.

Under paragraph 6 on page 3 of the Action, the Examiner states as a general rule: "It is well settled that in cases involving chemical and chemical compounds which differ radically in their properties it must appear in an applicant's specification

either by enumeration.....". It is absolutely not true that the different chemical compounds in the class of carbonic anhydrase inhibitors differ radically. As Dr. Stefansson points out, all of these molecules have the same basic structure which is responsible for the carbonic anhydrase inhibition, which is their common function, and they differ only in moieties which are responsible for differences in pharmacokinetic properties and distribution in the eye and body. The general rule by Dreshfield therefore does not apply here since it is unreasonable to state that these particular chemical compounds differ radically in their properties. Note also that in paragraph [0024] of the specification, the carbonic anhydrase inhibitors have been structurally described as heterocyclic or aryl sulfonamides and the generic and chemical names of eight representative CAIs useful herein have been enumerated. In the preceding paragraph [0023], numerous patents describing compounds of this type have been identified. Thus, carbonic anhydrase inhibitors are well-known in the art.

In statement 7 on page 4 of the Official Action, it is pointed out that the application only supplies clinical data from one carbonic anhydrase inhibitor, namely dorzolamide. While it is true that the clinical data comes just from dorzolamide, Dr. Stefansson notes that he and his co-workers have performed pharmacologic studies in animals that show that the pharmacologic action of vasodilatation and increased oxygenation which we believe is responsible for the influence on diabetic retinas is common with several carbonic anhydrase inhibitors such as acetazolamide, metazolamide, ethoxyzolamide and dorzolamide. (*Stefansson E, Pedersen DB, Jensen PK, la Cour M, Kiilgaard JF, Bang K, Eysteinsson T., Optic nerve oxygenation. Prog Retin Eye Res. 2005 May;24(3):307-32; Bach Pedersen D, Koch*

Jensen P, la Cour M, Kiilgaard JF, Eysteinsson T, Bang K, Wiencke AK, Stefansson E,Carbonic anhydrase inhibition increases retinal oxygen tension and dilates retinal vessels. Graefes Arch Clin Exp Ophthalmol. 2005 Feb;243(2):163-8 and Stefansson E, Jensen PK, Eysteinsson T, Bang K, Kiilgaard JF, Dollerup J, Scherfig E, la Cour M. Optic nerve oxygen tension in pigs and the effect of carbonic anhydrase inhibitors, Invest Ophthalmol Vis Sci. 1999 Oct;40(11):2756-61.) Copies of these references are enclosed. These studies have shown clearly that this relates to the carbonic anhydrase inhibition effect of these molecules and it is therefore reasonable to expect other carbonic anhydrase inhibitors to behave clinically in the same way as dorzolamide.

Regarding statement 8 on page 4 of the Action, it is well-known (Supuran et al 2003, Stefánsson et al 2005, both cited above) that the carbonic anhydrase inhibitors share a common fundamental structure, have the same pharmacologic action, that is, inhibition of carbonic anhydrase, influence retinal vessel diameter and oxygenation in the same way and have the same clinical effect such as lowering intraocular pressure and diureses. It is highly unlikely that they would share all these various structural and functional properties and yet behave differently than dorzolamide towards diabetic retinopathy. The principle is clear. Of course, a molecule that would be marketed for clinical use would have to be tested specifically, but its clinical effect is quite predictable from the available data.

In view of the foregoing, it is clear that the specification is fully enabling to one of ordinary skill in the art and that the 35 U.S.C. §112, first paragraph, rejection is unjustified and should be withdrawn.

Claims 6, 12-26, 31 and 37-51 have also been rejected under 35 U.S.C. §112, second paragraph, as indefinite. The Examiner states in this regard that "Claims 6,12-26, 31 and 37-51 are indefinite as to the expression slowing the progression of diabetic retinopathy in a diabetic not suffering from diabetes. The term, 'slowing the progression of diabetic retinopathy' indicates that the person already has had diabetic retinopathy." This statement, as noted by Dr. Stefansson in his declaration, involves a misunderstanding of the definition of diabetic retinopathy. Typically, a person who develops diabetes mellitus has no apparent ophthalmoscopical lesions in the retina for several years and during this time period the person is defined as having no retinopathy. Careful studies, such as the fluorophotometry as well as animal studies in diabetic animals demonstrate that diabetic retina in this stage has early changes which are simply not detectable by standard clinical examination such as fundus biomicroscopy and photography. The classification of no retinopathy is therefore based on the current ability for diagnosis and detection. It is a misunderstanding that this implies that nothing is going on in the retina and no development is going on. Quite to the opposite, clinical studies (DCCT study group: *Progression of retinopathy with intensive versus conventional treatment in the Diabetes Control and Complications Trial. Diabetes Control and Complications Trial Research Group. Ophthalmology. 1995 Apr;102(4):647-61.*) have demonstrated that the progress towards diabetic retinopathy can be influenced quite radically by controlling blood-glucose. Most diabetics present with microaneurisms in the capillaries of the retina as the first sign of diabetic retinopathy and this usually happens 5-15 years after the onset of diabetes mellitus. At this time they are classified as having diabetic retinopathy. This is also associated with capillary

occlusions in the retina. The carbonic anhydrase inhibitors dilate capillaries and Dr. Stefansson believes this is why they prevent capillary occlusions and thereby prevent the formation of microaneurisms and other signs of diabetic retinopathy. The instant treatment is aimed at preventing the complication of diabetes mellitus which is diabetic retinopathy and to do so before overt signs of diabetic retinopathy are present. It is simply a matter of classification to state that a diabetic has no retinopathy before the overt signs happen and then has retinopathy. Clearly this is a gradual continuous process ultimately marked by events which are visible and detectable by clinical examination. However, to further clarify the nature of this process, applicant has modified the language of the independent terms to recite "progression toward retinopathy" rather than "progression of retinopathy". Therefore, the 35 U.S.C. §112, second paragraph, rejection cannot rightly be maintained.

Claims 6, 12-26, 31 and 37-51 have been rejected under 35 U.S.C. 103(a) as purportedly being unpatentable over WO 99/44603 and Doshi et al. U. S. Patent No. 5,948,801.

The Doshi et al. patent deals with the treatment of retinal edema with brinzolamide. It is clear from the background of the invention that it is based on the premise that acetazolamide and other carbonic anhydrase inhibitors hasten the resorption of subretinal fluid, through an effect on the retinal pigment epithelium. Doshi et al. propose that brinzolamide may be useful in helping remove water from the edematous retina through its action on the retinal pigment epithelium. They specify all types of macular edema including that of diabetic retinopathy but they are certainly not suggesting brinzolamide as a treatment for diabetic retinopathy in

general. As Dr. Stefansson notes, such a claim would make no sense given the rationale and background of their invention.

And, in fact, they clearly state that retinal edema may develop in association with a variety of conditions, one of which is diabetic retinopathy (col. 1, lines 61-67); they never imply that they are treating the associated conditions, only retinal edema. In contrast, the present application is not claiming carbonic anhydrase inhibitors as treatment of macular edema. Rather, it is claiming carbonic anhydrase inhibitors as a treatment to slow down or prevent the progression towards diabetic retinopathy, which is completely different from retinal edema, even though a small minority of diabetics may develop retinal edema. Diabetic retinopathy is a potentially blinding disorder that affects the 150 million people that now have diabetes and will affect the 300 million people that will have developed diabetes within 20 years (*Zimmet P et al, Nature 414: 782-787, 2001*, copy enclosed). Less than 10% of those diabetics have macular edema (*Stefansson E, Bek T, Porta M, Larsen N, Kristinsson JK, Agardh E., Screening and prevention of diabetic blindness. Acta Ophthalmol Scand. 2000 Aug;78(4):374-85*, and other references cited in the accompanying declaration). Of the approximately 150 million diabetics in the world today, it can be estimated that 135 million do not have diabetic macular edema and approximately 75 million do not have visible diabetic retinopathy and are classified as having no retinopathy. None of these 75 million people would apply to the treatment as suggested by the patent by Doshi et al. Further, even when Doshi et al. suggest prevention of macular edema, it would presumably be on patients already having one of their associated conditions such as diabetic retinopathy. In the present case, however, the patient is a diabetic not yet showing signs of diabetic retinopathy. Therefore, the population is different.

Still further, a knowledgeable person would not draw the conclusion from the invention and patent of Doshi et al that carbonic anhydrase inhibitors would be useful for diabetic retinopathy in general. The diabetic retina is not edematous in the diabetic before he develops overt signs of diabetic retinopathy and also does not have edema in most cases after that. It is only the approximately the 10% of diabetics who have diabetic macular edema where it makes sense to increase the removal of water by affecting the pigment epithelium through carbonic anhydrase inhibition. This explains why Doshi et al did not make this jump and no one has in spite of the fact that the effect of carbonic anhydrase inhibition on the subretinal fluid resorption by acetazolamide has been known for more than 20 years (*Marmor et al, Investigative of Ophthalmology 121-124, 1982*; see the background of invention by Doshi et al.) A knowledgeable person would indeed made the distinction between diabetic macular edema (and retinal edema in general) and diabetic retina with no retinopathy as well as the diabetic retina with early non-proliferative diabetic retinopathy. The difference between the two would be obvious to a person skilled in the art. Based on the background and invention by Doshi et al, it would make absolutely no sense at all to propose that this would be useful in slowing the development towards diabetic retinopathy in a diabetic.

WO 99/44603 (Sponsel) relates to a composition and method for treating certain ocular disorders, particularly macular edema and macular degeneration, by applying a topical carbonic anyhdrase inhibitor and an ocular hypotensive agent or inotropic agent in an amount sufficient to improve visual function. Thus, Sponsel uses a combination of active agents to achieve treatment of macular disorders and improve visual function. The present applicant does neither. As noted above,

macular edema develops in association with certain conditions, including retinitis pigmentosa and diabetic retinopathy. Sponsel never suggest treatment of the underlying conditions, only of the macular edema which develops therewith. Thus, the points made with respect to Doshi et al. above are equally valid with respect to Sponsel. Most especially, it is again pointed out that even if Sponsel is taken as suggesting use of his agents to prevent macular edema, the patients would already have manifested the associated conditions, e.g., diabetic retinopathy. However, in the present invention, the patient is diabetic but has not yet manifested signs of diabetic retinopathy. Therefore, the population is different. Sponsel proposes a treatment for retinal edema. He points out that a number of diseases may cause retinal (macular) edema, including retinitis pigmentosa, branch retinal vein occlusion and diabetic retinopathy. As Dr. Stefansson notes, he does not suggest and a knowledgeable person would not reason that he is proposing treatment for retinitis pigmentosa or diabetes mellitus. His patent is 5 years old and neither Sponsel nor anybody else has suggested that the Sponsel references extend beyond the treatment of retinal edema.

Moreover, as Dr. Stefansson also points out in his declaration, the patents by Doshi et al and Sponsel do not give a rationale and do not suggest to the knowledgeable person that their treatment would be useful for diabetic retinopathy in general, any more than it would be useful for retinitis pigmentosa in general. It would be stretching the point even further to suggest that they are proposing drug treatment that should be used for diabetics who do not have retinopathy. No knowledgeable person would make that connection based on the patent and the background by Doshi et al and Sponsel. Indeed the entire field of knowledgeable

persons including Drs. Doshi and Sponsel have had the opportunity to do so over at least 5 years and have not done so and this strongly supports Dr. Stefansson's claim that this connection cannot be made based on the data and thesis set forth in their patents.

Furthermore, present Claim 31 and its dependent Claims 37-51 require that the carbonic anhydrase inhibitor be the sole agent administered to slow the progression toward diabetic retinopathy, while Sponsel's method requires a combination of a carbonic anhydrase inhibitor and another active agent for his treatment of retinal edema.

In view of the foregoing, it is submitted that all of applicant's claims are free of the §103 rejection based on WO99/44603 (Sponsel) and Doshi et al.

In light of the above, all record rejections are believed to be overcome.

Further, favorable action in the form of a Notice of a Allowance is believed to be next in order and is earnestly solicited.

Respectfully submitted,

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Carbonic Anhydrase Inhibitors

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Abstract: At least 14 different carbonic anhydrase (CA, EC 4.2.1.1) isoforms were isolated in higher vertebrates, where these zinc enzymes play crucial physiological roles. Some of these isozymes are cytosolic (CA I, CA II, CA III, CA VII), others are membrane-bound (CA IV, CA IX, CA XII, and CA XIV), CA V is mitochondrial and CA VI is secreted in saliva. Three acatalytic forms are also known, which are denominated CA related proteins (CARP), CARP VIII, CARP X, and CARP XI. Several important physiological and physio-pathological functions are played by many CA isozymes, which are strongly inhibited by aromatic and heterocyclic sulfonamides as well as inorganic, metal complexing anions. The catalytic and inhibition mechanisms of these enzymes are understood in detail, and this helped the design of potent inhibitors, some of which possess important clinical applications. The use of such enzyme inhibitors as antiglaucoma drugs will be discussed in detail, together with the recent developments that led to isozyme-specific and organ-selective inhibitors. A recent discovery is connected with the involvement of CAs and their sulfonamide inhibitors in cancer: several potent sulfonamide inhibitors inhibited the growth of a multitude of tumor cells *in vitro* and *in vivo*, thus constituting interesting leads for developing novel antitumor therapies. Furthermore, some other classes of compounds that interact with CAs have recently been discovered, some of which possess modified sulfonamide or hydroxamate moieties. Some sulfonamides have also applications as diagnostic tools, in PET and MRI or as antiepileptics or for the treatment of other neurological disorders. Future prospects for drug design applications for inhibitors of these ubiquitous enzymes are also discussed. Med Res Rev, 23, No. 2, 146–189, 2003

Key words: carbonic anhydrase; isozymes; sulfonamide; X-ray; glaucoma; anticanceragent; diagnostic tool

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1. INTRODUCTION

The carbonic anhydrases (CAs, EC 4.2.1.1) are ubiquitous zinc enzymes, present in *Archaea*, prokaryotes and eukariotes, being encoded by three distinct, evolutionarily unrelated gene families: the α-CAs (present in vertebrates, eubacteria, algae and cytoplasm of green plants), the β-CAs (predominantly in eubacteria, algae and chloroplasts of both mono- as well as dicotyledons) and the γ-CAs (mainly in *Archaea* and some eubacteria), respectively.[1–3] In higher vertebrates, including humans, 14 different CA isozymes or CA-related proteins (CARP) have been described (Table I), with very different subcellular localization and tissue distribution.[1–3] Basically, there are several cytosolic forms (CA I–III, CA VII), four membrane-bound isozymes (CA IV, CA IX, CA XII, and CA XIV), one mitochondrial form (CA V), as well as a secreted CA isozyme, CA VI.[1,2] These enzymes catalyze a very simple physiological reaction, the interconversion between carbon dioxide and the bicarbonate ion, and are thus involved in crucial physiological processes connected with respiration and transport of CO_2/bicarbonate between metabolizing tissues and lungs, pH and CO_2 homeostasis, electrolyte secretion in a variety of tissues/organs, biosynthetic reactions (such as gluconeogenesis, lipogenesis and ureagenesis), bone resorption, calcification, tumorigenicity, and many other physiologic or pathologic processes.[1–3] As will be discussed shortly, many of these isozymes are important targets for the design of inhibitors with clinical applications.

In addition to the physiological reaction, the reversible hydration of CO_2 to bicarbonate (reaction 1, Fig. 1), CAs catalyze a variety of other reactions, such as: the hydration of cyanate to carbamic acid, or of cyanamide to urea (reactions 2 and 3); the aldehyde hydration to *gem*-diols (reaction 4); the hydrolysis of carboxylic, or sulfonic (reactions 5, 6), as well as other less investigated hydrolytic processes, such as those described by Equations 7–9 in Figure 1.[2,4–6] It should be mentioned that the previously reported phosphatase activity of CA III was recently proved to be an artefact.[7] It is unclear at this moment whether CA catalyzed reactions other than the CO_2 hydration have physiological significance. The X-ray crystal structure has been determined for six

Table I. Higher Vertebrate α-CA Isozymes, Their Relative CO_2 Hydrase Activity, Affinity for Sulfonamide Inhibitors, and Sub-Cellular Localization

Isozyme	*Catalytic activity (CO_2 hydration)*	*Affinity for sulfonamides*	*Sub-cellular localization*
CA I	Low (10% of that of CA II)	Medium	Cytosol
CA II	High	Very high	Cytosol
CA III	Very low (0.3% of that of CA II)	Very low	Cytosol
CA IV	High	High	Membrane-bound
CA V	Moderate-high[a]	High	Mitochondria
CA VI	Moderate	Medium-low	Secreted into saliva
CA VII	High	Very high	Cytosol
CARP VIII	Acatalytic	*	Probably cytosolic
CA IX	High	High	Membrane-bound
CARP X	Acatalytic	*	Unknown
CARP XI	Acatalytic	*	Unknown
CA XII	Active (no quantitative data)	Unknown	Membrane-bound
CA XIII[b]	Probably high	Unknown	Unknown
CA XIV	Low	Unknown	Membrane-bound

[a]Moderate at pH 7.4, high at pH 8.2 or higher.

[b]CA XIII has not been isolated as a protein but has been identified from an expressed sequence tag (EST) derived from a mouse mammary gland cDNA library.[3]

*The native CARP isozymes do not contain Zn(II), so that their affinity for the sulfonamide inhibitors has not been measured. By site-directed mutagenesis it is possible to modify these proteins and transform them in enzymes with CA-like activity which probably are inhibited by sulfonamides, but no studies on this subject are available presently.

$O=C=O + H_2O \Leftrightarrow HCO_3^- + H^+$	(1)
$O=C=NH + H_2O \Leftrightarrow H_2NCOOH$	(2)
$HN=C=NH + H_2O \Leftrightarrow H_2NCONH_2$	(3)
$RCHO + H_2O \Leftrightarrow RCH(OH)_2$	(4)
$RCOOAr + H_2O \Leftrightarrow RCOOH + ArOH$	(5)
$RSO_3Ar + H_2O \Leftrightarrow RSO_3H + ArOH$	(6)
$ArF + H_2O \Leftrightarrow HF + ArOH$ (Ar = 2,4-dinitrophenyl)	(7)
$PhCH_2OCOCl + H_2O \Leftrightarrow PhCH_2OH + CO_2 + HCl$	(8)
$RSO_2Cl + H_2O \Leftrightarrow RSO_3H + HCl$ (R = Me; Ph)	(9)

Figure 1. Reactions catalyzed by α-CAs.

α-CAs at this moment (isozymes CA I–V and CA XII),[8] as well as for representatives of the β- and γ-CA families.[9]

2. *CATALYTIC AND INHIBITION MECHANISMS OF CARBONIC ANHYDRASES*

The Zn(II) ion of CAs is essential for catalysis.[1,2,7] X-ray crystallographic data showed that the metal ion is situated at the bottom of a 15 Å deep active site cleft (Fig. 2), being coordinated by three histidine residues (His 94, His 96, and His 119) and a water molecule/ hydroxide ion.[1,2,7,10] The zinc-bound water is also engaged in hydrogen bond interactions with the hydroxyl moiety of Thr 199, which in turn is bridged to the carboxylate moiety of Glu 106; these interactions enhance the nucleophilicity of the zinc-bound water molecule, and orient the substrate (CO_2) in a favorable location for the nucleophilic attack (Fig. 3).[2,7,10–12] The active form of the enzyme is the basic one, with hydroxide bound to Zn(II) (Fig. 3A).[12] This strong nucleophile attacks the CO_2 molecule bound in a hydrophobic pocket in its neighbourhood (the elusive substrate-binding site comprises residues

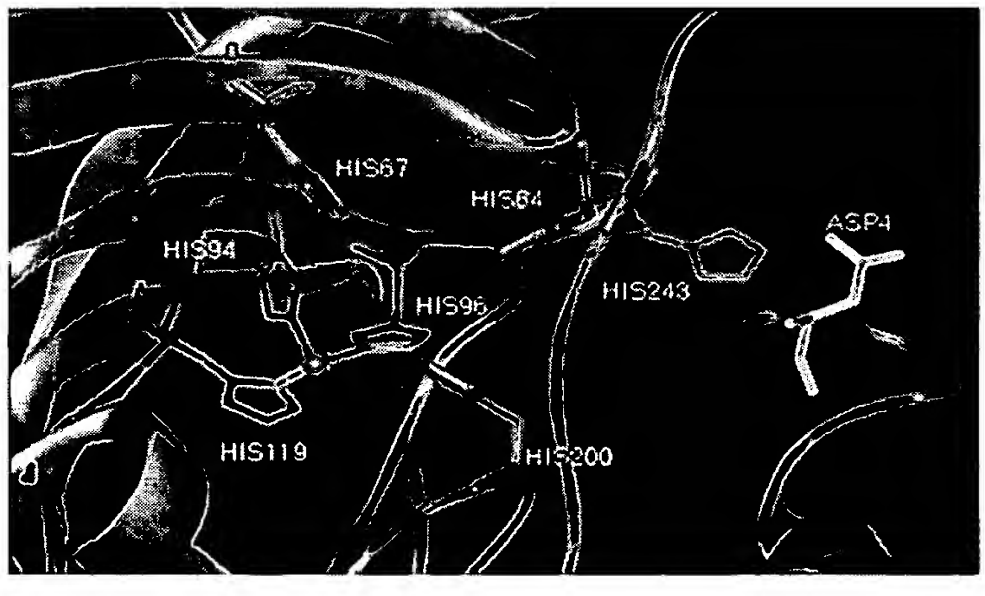


Figure 2. hCA I active site, with the Zn(II) ion coordinated by three histidine ligands (His 94, His 96, and His 119, in green), the proton shuttle residue His 64 as well as other residues important for the catalytic cycle and the binding of substrates/inhibitors, such as His 67, His 200, His 243, and Asp 4.[10]

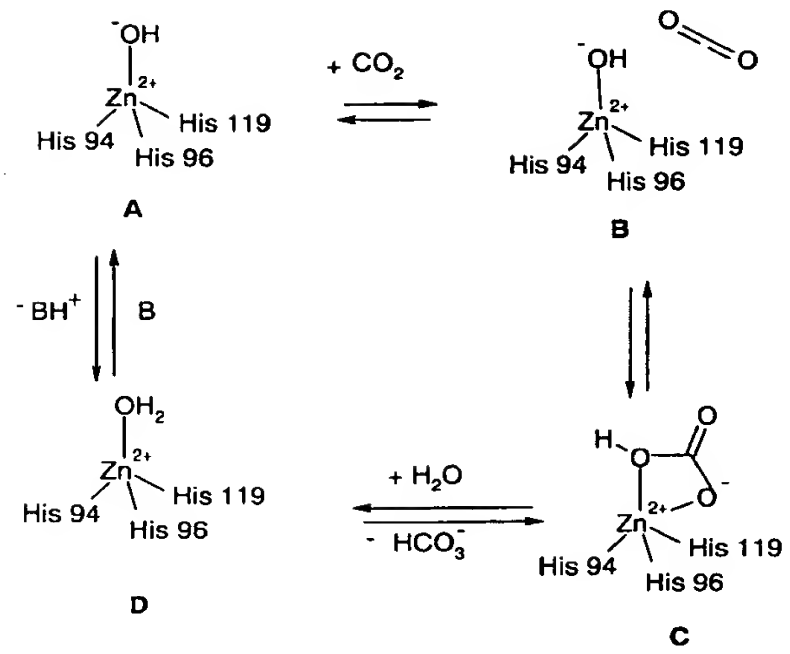


Figure 3. Schematic representation of the catalytic mechanism for the CA catalyzed CO_2 hydration.

Val 121, Val 143, and Leu 198 in the case of the human isozyme CA II[11]) (Fig. 3B), leading to the formation of bicarbonate coordinated to Zn(II) (Fig. 3C). The bicarbonate ion is then displaced by a water molecule and liberated into solution, leading to the acid form of the enzyme, with water coordinated to Zn(II) (Fig. 3D), which is catalytically inactive.[2,7,10] In order to regenerate the basic form A, a proton transfer reaction from the active site to the environment takes place, which may be assisted either by active site residues (such as His 64—the proton shuttle in isozymes CA I and II, see Fig. 2) or by buffers present in the medium. The process may be schematically represented by Equations (10) and (11) below:

$$EZn^{2+}\text{-}OH^- + CO_2 \Longleftrightarrow EZn^{2+}\text{-}HCO_3^- \Longleftrightarrow EZn^{2+}\text{-}OH_2 + HCO_3^- \quad (10)$$

$$EZn^{2+}\text{-}OH_2 \Longleftrightarrow EZn^{2+}\text{-}HO^- + H^+ \quad (11)$$

The rate limiting step in catalysis is the second reaction, i.e., the proton transfer that regenerates the zinc-hydroxide species of the enzyme.[12] In the catalytically very active isozymes, such as CA II, CA IV, CA V, CA VII, and CA IX, the process is assisted by a histidine residue placed at the entrance of the active site (His 64), as well as by a cluster of histidines, which protrudes from the rim of the active site to the surface of the enzyme, assuring thus a very efficient proton transfer process.[10] This also explains why CA II is one of the most active enzymes known (with a $k_{cat}/K_m = 1.5 \times 10^8\ M^{-1}sec^{-1}$), approaching the limit of diffusion control,[7-12] and also has important consequences for the design of inhibitors with clinical applications.

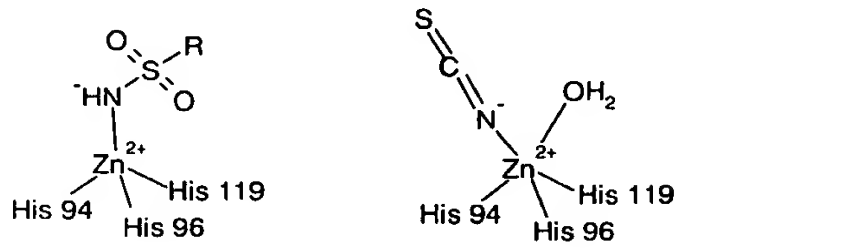
Two main classes of CA inhibitors (CAIs) are known: the metal complexing anions, and the unsubstituted sulfonamides, which bind to the Zn(II) ion of the enzyme either by substituting the non-protein zinc ligand (Eq. 12 in Fig. 4) or add to the metal coordination sphere (Eq. 13 in Fig. 4), generating trigonal-bipyramidal species.[2,7,13,14] Sulfonamides, which are the most important CAIs, bind in a tetrahedral geometry of the Zn(II) ion (Fig. 4A), in deprotonated state, with the nitrogen atom of the sulfonamide moiety coordinated to Zn(II); anions may bind either in tetrahedral geometry of the metal ion or as trigonal-bipyramidal adducts, such as for instance the thiocyanate adduct shown in Figure 4B.[15]

$$\text{E-Zn}^{2+}\text{-OH}_2 + \text{I} \Leftrightarrow \text{E-Zn}^{2+}\text{-I} + \text{H}_2\text{O} \qquad \textbf{(substitution)} \qquad (12)$$

Tetrahedral adduct

$$\text{E-Zn}^{2+}\text{-OH}_2 + \text{I} \Leftrightarrow \text{E-Zn}^{2+}\text{-OH}_2\text{(I)} \qquad \textbf{(addition)} \qquad (13)$$

Trigonal-bipyramidal adduct



Tetrahedral adduct (sulfonamide) **A**

Trigonal-bipiramidal adduct (thiocyanate) **B**

Figure 4. CA inhibition mechanism by sulfonamide and anionic inhibitors.

X-ray crystallographic structures are available for many adducts of sulfonamide inhibitors with isozymes CA I, II, and IV.[2,7,15–17] In all these adducts, the deprotonated sulfonamide is coordinated to the Zn(II) ion of the enzyme, and its NH moiety participates in a hydrogen bond with the Oγ of Thr 199, which in turn is engaged in another hydrogen bond to the carboxylate group of Glu 106.[15–17] One of the oxygen atoms of the SO_2NH moiety also participates in a hydrogen bond with the backbone NH moiety of Thr 199.[15–17] In Figure 5, the crystal structures of the hCA II adducts with the simplest compounds incorporating a sulfamoyl moiety (sulfamide and sulfamic acid, respectively) are shown. The bi-negatively charged $(NH)SO_3^{2-}$ sulfamate ion and the monoanion of sulfamide $NHSO_2NH_2^-$ were shown to bind to the Zn(II) ion within the enzyme active site.[16] These two structures provide some close insights into why this functional group (the sulfonamide one) appears to have unique properties for CA inhibition: (i) it exhibits a negatively charged, most likely mono-protonated nitrogen coordinated to the Zn(II) ion; (ii) simultaneously this group forms a hydrogen bond as donor to the oxygen Oγ of the adjacent Thr 199, and (iii) a hydrogen bond is formed between one of the SO_2 oxygens to the backbone NH of Thr 199. Thus, the basic structural elements explaining the strong affinity of the sulfonamide moiety for the Zn(II) ion of CAs were delineated in all details by using these simple compounds as prototypical CAIs, without the need to analyze the interactions of the organic scaffold usually present in other inhibitors (generally belonging to the aromatic/heterocyclic sulfonamide class[16]). Despite important similarities for the binding of these two inhibitors to the enzyme with that of aromatic/heterocyclic sulfonamides of the type RSO_2NH_2 previously investigated, the absence of a $C\text{-}SO_2NH_2$ bond in sulfamide/sulfamic acid, leads to a different hydrogen bond network in the neighborhood of the catalytical Zn(II) ion, which was shown to be useful for the drug design of more potent CA inhibitors, possessing different zinc binding functions than the classical sulfonamide one.[16]

The physiological function of the major red cell isozyme, CA I (present in concentrations of up to 150 μM in the blood)[1,2] is still unknown. Recently, the X-ray crystal structure of a natural mutant of CA I, i.e., CA I Michigan 1 (Fig. 6) was reported (this isozyme was isolated in three generations of a family of European Caucasians[18]). CA I Michigan 1 differs from wild type CA I in a single amino acid residue present within the active site cavity, i.e., Arg 67 instead of His 67.[18] This amino acid residue is located in an important region of the catalytic site, which is involved both in shuttling protons

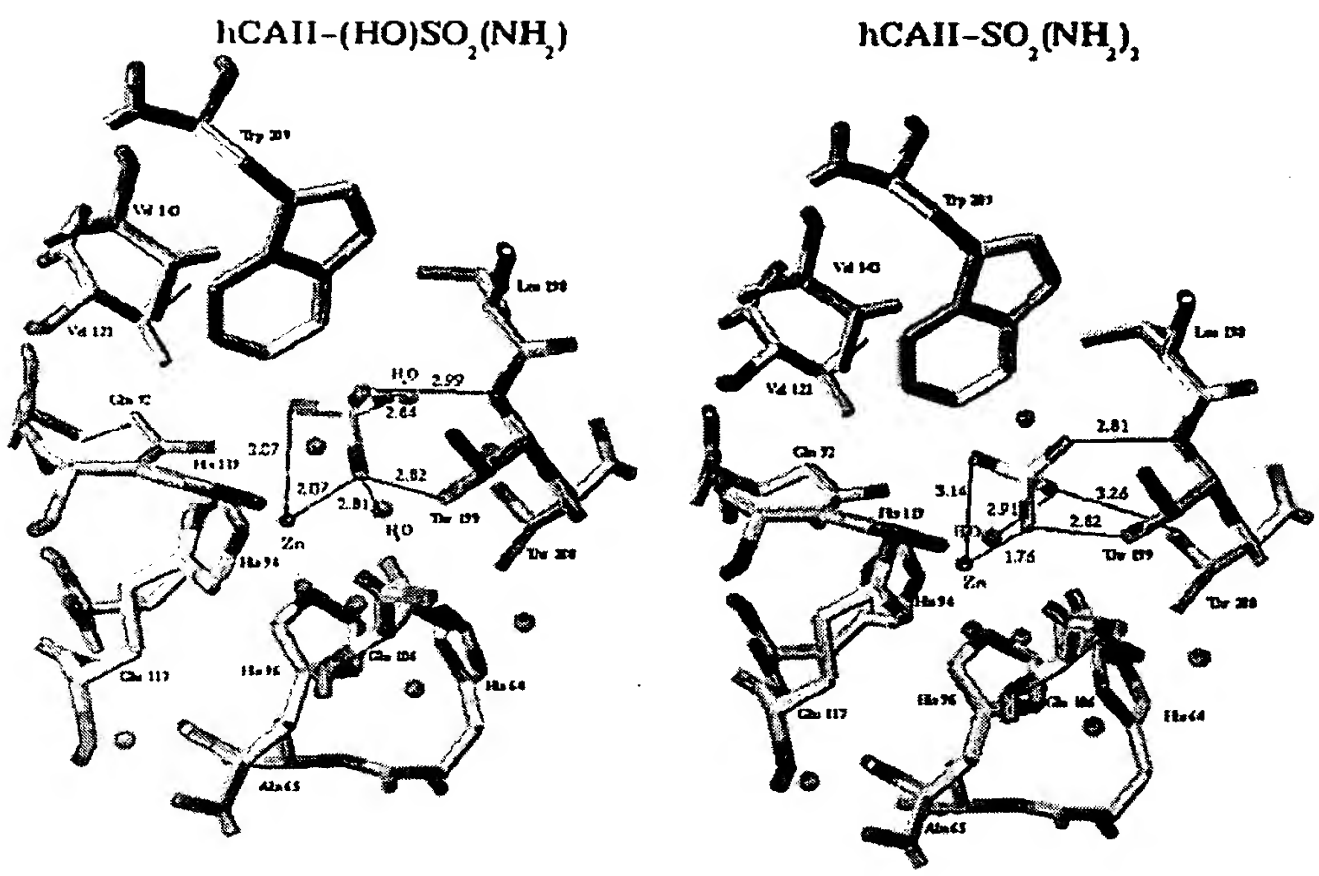


Figure 5. Adducts of hCA II with the simplest sulfonamides: sulfamic acid H_2NSO_3H, (left) and sulfamide $H_2NSO_2NH_2$ (right), determined by X-ray crystallography.[16]

from the active cavity to the environment and also in the binding of aromatic/heterocyclic sulfonamides, the classical, clinically important CAIs (Fig. 6). The structure of the native mutant enzyme as well as its adduct with a second zinc ion has been determined, which revealed the presence of a second metal ion binding site within the active cavity. Arg 67 appeared to promote the binding of

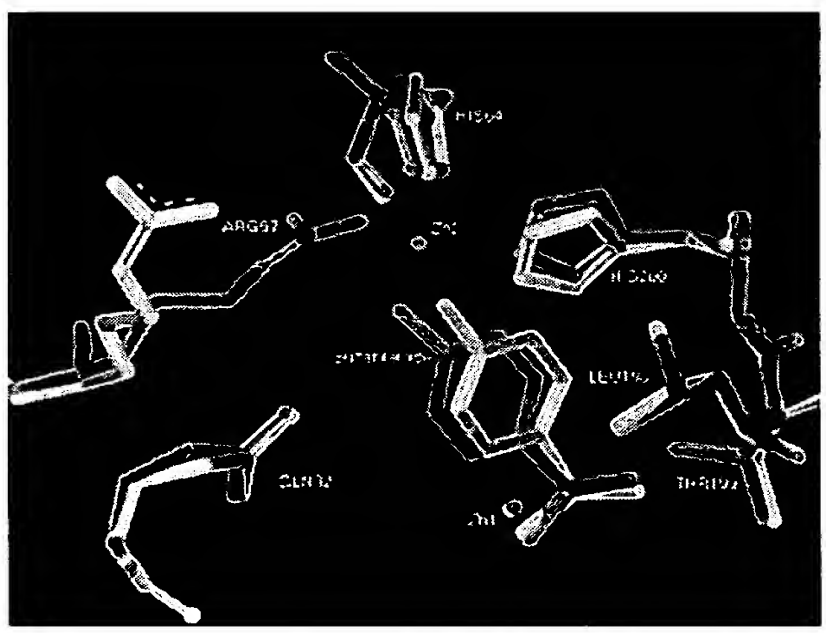
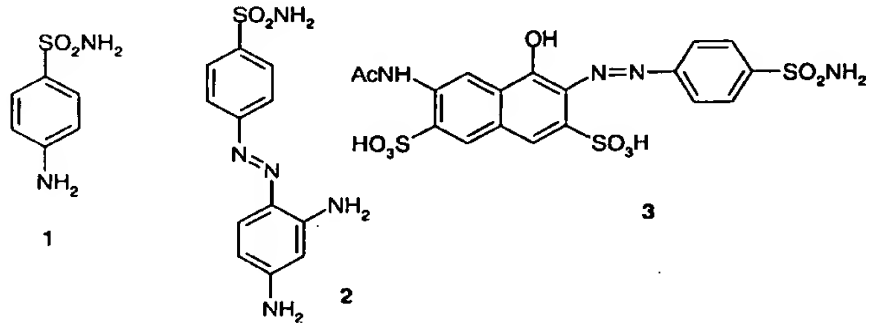


Figure 6. Least-squares superimposition of the most relevant active site residues of the natural mutant CA I Michigan 1 (in yellow) and the CA I Michigan 1 $(Zn)_2$ adduct (in red), involved in sulfonamide inhibitor binding, with bound sulfanilamide, as determined by X-ray crystallography. The catalytic zine ion is Zn1.[18]

this second zinc ion to His 64, His 200, and itself (through one of the guanidino nitrogen atoms).[18] This second metal ion bound into the active cavity is involved in the previously observed activation mechanism for substrate specific α- and β-naphthyl acetates hydrolyses.[18] Furthermore, this is the first example of a Zn(II) enzyme containing an arginine residue in the metal ion coordination sphere, as well as the first CA isozyme that binds two metal ions within its active site.[18] The crystal structures of sulfanilamide (4-aminobenzene-sulfonamide) complexed to native hCA I Michigan 1 variant and to its $(Zn)_2$ adduct were also reported.[19] Comparisons among these structures and the corresponding sulfonamide adduct of hCA I evidenced significant differences in the orientation of the inhibitor molecule and in its interactions with active site residues such as His 200, Thr 199, Leu 198, Gln 92, and Arg/His67 which are known to play important roles in substrate or inhibitor binding and recognition.[1,2,18,19] In CA I Michigan 1, it was observed a lengthening of the Zn-N1 sulfanilamide bond distance and a corresponding shortening of the distance between the sulfamido group and Thr 199 with respect to wild type CA I. When the second Zn(II) ion was present within the active site, the *p*-amino group and the aromatic ring of the inhibitor molecule appeared tilted towards Gln 92 and Arg 67, moving away from residues His 200 and Leu 198. The structural differences in inhibitor binding between the CA I isozyme and the CA I Michigan 1 variant showed that even a point mutation within the active site of a CA isozymes may have relevant consequences for the binding of inhibitors.[19] This work opens new ways for the design of isozyme-specific CA inhibitors.

3. CA INHIBITORS IN DRUG DESIGN

CA inhibition with sulfanilamide (**1**) discovered by Mann and Keilin[20] was the beginning of a great scientific adventure that led to important drugs, such as the antihypertensives of benzothiadiazine and high-ceiling diuretics type,[21] the sulfonamides with CA inhibitory properties mainly used as antiglaucoma agents,[2,21,22] some antithyroid drugs,[21] the hypoglycemic sulfonamides[23] and, ultimately, some novel types of anticancer agents.[24]

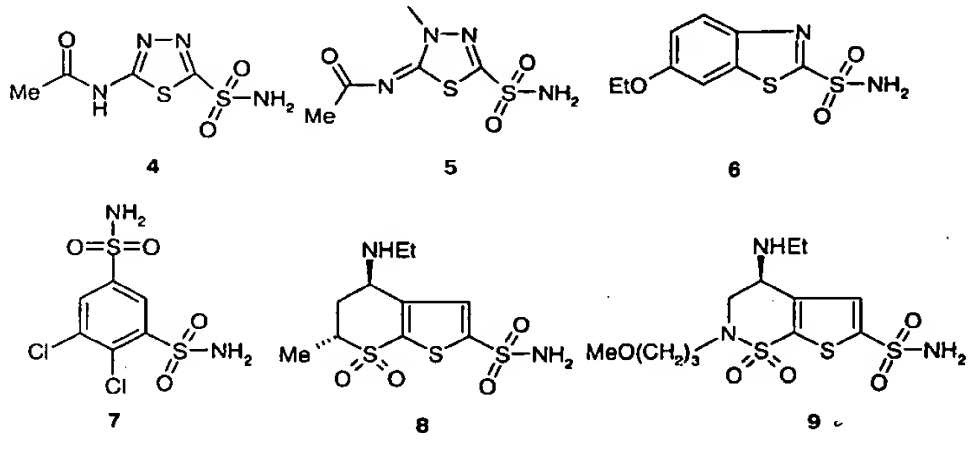


The report of Krebs[25] that mainly the unsubstituted aromatic sulfonamides of type $ArSO_2NH_2$ act as strong CAIs, and that the potency of such compounds is drastically reduced by *N*-substitution of the sulfonamide moiety, constituted the beginning of extensive structure–activity correlations, which led to some valuable drugs during a short period of time. Among the active structures found by Krebs were also the azodyes **2** (prontosil red) and **3**, derived from sulfanilamide. The early stages of CAIs development have thouroughly been reviewed by Maren,[21,22] whereas literature till 1993 was reviewed by Supuran[26] and more incompletely (up to 1996) by Mansoor et al.[27] Thus, in this review we will concentrate on the recent developments in this field that led to important advances in the design of topically acting antiglaucoma sulfonamides, isozyme-specific inhibitors, inhibitors with

modified sulfonamide moieties, antitumor sulfonamides, as well as diagnostic tools and biosensors based on this class of pharmacological agents.

A. Topically Acting Sulfonamides as Antiglaucoma Drugs

Four systemic sulfonamide drugs have been used clinically, mainly as antiglaucoma agents, for a long time: acetazolamide (**4**), methazolamide (**5**), ethoxzolamide (**6**), and dichlorophenamide (**7**).[2,21] Systemic inhibitors are useful in reducing elevated intraocular pressure (IOP) characteristic to this disease, as they represent the most efficient physiological treatment of glaucoma. This is because by inhibiting the ciliary process enzyme (the sulfonamide susceptible isozymes CA II and CA IV), a reduced rate of bicarbonate and aqueous humor secretion is achieved, which leads to a 25–30% decrease of IOP, but the inhibition of various CA isozymes present in tissues other than that of the eye,



4 **5** **6**

7 **8** **9**

leads to an entire range of side effects.[2,21] The most proeminent ones are: numbness and tingling of extremities, metallic taste, depression, fatigue, malaise, weight loss, decreased libido, gastrointestinal irritation, metabolic acidosis, renal calculi and transient myopia.[2,21] Indeed, as seen from data of Table II, compounds **4–7** indiscriminately inhibit several CA isozymes (such as CA I, CA II, CA IV, CA V, and CA VII) abundant in organs other than the eye, such as blood, kidneys, lungs, gastrointestinal tract, CNS, etc.[2,21]

Table II. Inhibition Data for the Clinically Used Sulfonamides **4–9** Against Several α-CA Isozymes

	K_I (nM)					
Isozyme	**4**	**5**	**6**	**7**	**8**	**9**
hCA I	200	10	1	350	50,000	nt
hCA II	10	8	0.7	30	9	3
hCA III	3×10^5	1×10^5	5×10^3	nt	8×10^3	nt
hCA IV	66	56	13	120	45	45
mCA V	60	nt	5	nt	nt	nt
hCA VI	1,100	560	nt	nt	nt	nt
mCA VII	16	nt	0.5	nt	nt	nt

h, human; m, murine isozyme; nt, not tested (no data available).

It appeared thus of interest to try to administer such sulfonamide CAIs topically, directly into the eye.[2,21] Still, none of the clinically used inhibitors **4–7** (or other structurally related investigational agents)[21] proved to be effective when administered topically in reducing elevated IOP, and thus, for a long period, it was considered that sulfonamide CAIs cannot be administered *via* topical route.[22] In a classical study, Maren's group then showed that this is principally due to the unappropriate physico-chemical properties of the existing CAIs available at that moment, and put the basis for the discovery of the topically acting sulfonamides: the first such drug, dorzolamide (**8**),[29] entered in clinics in 1995, and the second one (structurally related to dorzolamide)-brinzolamide (**9**)[30] entered in clinics in 1999. Thus, in addition to the systemically acting inhibitors **4–7**, the clinical antiglaucoma armamentarium comprises these two new drugs, which show much less side effects as compared to the first drugs mentioned above, but which also basically inhibit all the physiologically relevant CA isozymes (Table II).[2]

Two main approaches were used for the drug design of effective antiglaucoma sulfonamides.

The "ring" approach, used for the discovery of dorzolamide and brinzolamide, consisted in exploring a great variety of ring systems on which the sulfonamide group (and eventually other moieties) have been attached. This approach was really beneficial for the chemistry of this class of derivatives—as a very large number of new ring systems was explored in this way, but generally led to few effective *in vivo* IOP lowering agents (except for the two drugs mentioned above), and the largest majority of the obtained derivatives were potent allergens.

The second approach, the "tail" one, consisted in attaching water-solubilizing tails to different scaffolds of well-known aromatic/heterocyclic sulfonamides possessing affinity for the CA active site, assuring in this way the possibility to modulate in greater details the physico-chemical properties of these pharmacological agents. This approach did not lead for the moment to a clinically used drug (it was reported relatively recently), but led to the development of a very large number of much more effective antiglaucoma sulfonamides as compared to **8** and **9** (at least in animal models of the disease) in comparison with the ring approach. One must also mention that these two aproaches are both valuable, and complementary, since it was proved for example that dorzolamide may also be further derivatized by the tail strategy, leading to even more effective IOP lowering compounds (see later in the text).

B. The "Ring" Approach

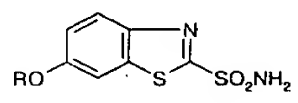
The lead molecule used for the development of dorzolamide (**8**), and subsequently brinzolamide (**9**), was undoubtedly ethoxzolamide (**6**), a very strong CAI (Table II). Indeed, the largest majority of the sulfonamides designed by the ring approach are bicyclic, with at least one of the two rings containing a sulfur atom.

The first candidates belonging to this family of sulfonamides, investigated in some details, were just *O*-acyl- or *O*-alkylsulfonyl derivatives of 6-hydroxy-benzothiazole-2-sulfonamide of type **10**, reported by Woltersdorf et al.[31] Many of these derivatives were very effective (in the low nanomolar range) CA II inhibitors, and acted as ocular prodrugs: their ester moiety was hydrolyzed *in situ* by esterases present in the eye tissues, liberating the free phenol (**10**, R = H) that assured a relatively effective IOP lowering.[31] Still, these compounds could not be developed for clinical use for two reasons: they were rapidly metabolized, with the sulfonamido group being nucleophilically displaced by reduced glutathione, and on the other hand, they proved to be very potent allergens *in vivo*, in rabbits (a property compound **10** shares with a number of related bicyclic sulfonamides that will be discussed shortly).[31] The allergic reactions were considered to be due to arylation of biological macromolecules promoted by the heterocyclic sulfonamides, leading subsequently to immune responses.[31]

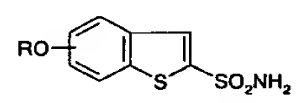
The above mentioned side effects of the benzothiazole-2-sulfonamides **10** were considered to be mainly due to the highly electrophilic character of the heterocyclic ring, and thus the next step consisted in reducing this electrophilicity, by designing sulfonamides incorporating other ring

systems, such as benzo[b]thiophene-,[32] benzofuran-,[33] indole-,[33] and thieno-thiophene-,[34] with derivatives of types **11–15** investigated in some detail.

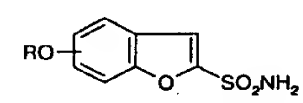
These derivatives (**11–15**) generally showed very good *in vitro* CA inhibitory properties against isozyme hCA II, and some of them also showed modest, but interesting IOP lowering properties when administered topically in normotensive or glaucomatous rabbits, but many of them (mainly the benzofuran- and indole-2-sulfonamide derivatives) were still potent allergens, and were ineffective in humans.[33] Less allergenic effects showed the benzo[b]thiophene-2-sulfonamide **11**,[32] and the thieno-thiophene-2-sulfonamides **14**, **15**,[34] but these latter derivatives showed a strong binding to the ocular pigment, in addition to a modest reactivity towards glutathione, which were considered not really desirable properties for a future drug. Thus, such compounds were not appropriate candidates for the development of novel topically acting sulfonamide CAIs, also because generally they possessed a very poor water solubility, so that it had to be formulated as suspensions.



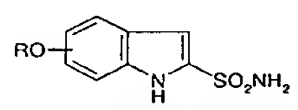
10: R = acyl, alkyl-SO_2



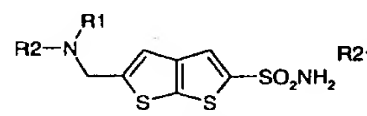
11: R= 5-/6-substituted-acyl, H



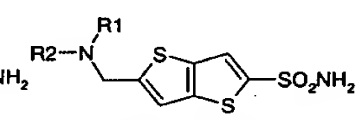
12: R= 5-/6-substituted-acyl, H



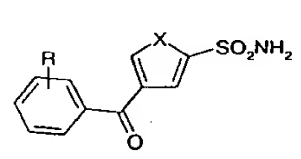
13: R= 5-/6-substituted-acyl, H



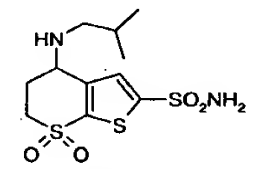
14: R1, R2 = H, alkyl, etc.



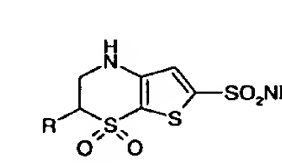
15: R1, R2 = H, alkyl, etc.



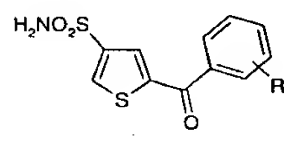
16: X = S, R = OH, OMe, etc.
17: X = O, R = OH, OMe, etc.



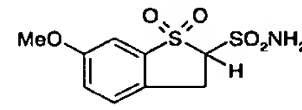
18: MK-927



19: R=CH_2CH_2NR1R2



20: R = H, alkyls, MeO, etc.



21

22: X = Cl, Y = H
23: X = F, Y = H
24: X = Y = Cl
25: X = Me, Y = H

CF_3-SO_2NH_2

26

C_nX_{2n+1}- SO_2NH_2

27: n = 1-4, X = F, Cl

A slightly different approach was reported by the same group,[35] and consisted in preparing a series of 4-substituted-thiophene-and -furan-2-sulfonamide derivatives of types **16** and **17**. These compounds showed good CA inhibitory properties and interesting *in vivo* IOP lowering effects in rabbits, being also devoid of allergenic properties. In fact, they are the most similar among the investigated sulfonamides of Merck with the compounds that led to the successful inhibitor **8**, belonging to the thieno[2,3-b]thiopyran-2-sulfonamide class. Indeed, sulfonamides belonging to this ring system were already reported in 1987 by Ponticello et al.,[36] and they were among the most water soluble sulfonamides reported at that time. By varying the substituent in the 4-position of the heterocyclic ring, MK-927 (**18**) was initially considered the best candidate for clinical development,[37] but subsequently the presence of a new chiral center was considered desirable, and that ultimately led to dorzolamide (**8**).[37]

Similarly to the other bicyclic sulfonamides reported by the Merck group, thienothiopyran sulfonamide derivatives (such as **8**, **18**, and many other representatives synthesized during these valuable studies)[36,37] have nanomolar affinity for the critical isozyme in aqueous humor secretion

(hCA II), and in contrast to all other investigated sulfonamides, they possess good water solubility (as hydrochloride salts), in the range of 1–2%! This property is obviously due to the presence of secondary amine moieties in their molecules, and although it constituted the first important success in the design of topically acting sulfonamides, it later became the source of discontent with dorzolamide: the acidic pH of its ophthalmologic solutions (pH 5.5) led to side effects including stinging, burning or reddening of the eye, blurred vision, and pruritus.[2,29] Although the Merck group investigated some thiazine-thiopyransulfonamides of type **19**, after the report of dorzolamide-like derivatives,[38] this defect of the drug was not corrected. In fact, it is not clear what advantage the compounds **19** might have over the structurally related thieno-thiopyransulfonamides.

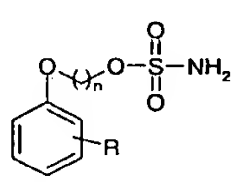
It is interesting to note that probably by using derivatives **16** as leads, an Allergan group reported[39] a large series of 5-substituted-3-thiophenesulfonamides of type **20**, which generally showed good CA inhibitory properties, but no topical activity in a rabbit glaucoma model.

An interesting variant of the "ring" aproach has also been reported by the Merck group, using the benzo[b]thiophene-2-sulfonamide derivatives **11** as leads.[40] Thus, borohydride hydrogenation of one derivative of type **11** led to the "aliphatic"-like sulfonamide **21**, which still showed good CA inhibitory properties (at that time it was generally considered that aliphatic sulfonamides are devoid of such properties).[21] This compound was then used as lead, and generated really aliphatic derivatives of types **22–25**, which showed nanomolar affinity towards CA II, with one compound (the fluoroderivative **23**) possessing *in vivo* IOP lowering effects in rabbits comparable to those of MK-927.[40]

Two strictly related approaches with the above-mentioned one were reported by Maren's group[41,42] and although they do not constitute clear-cut examples of the "ring approach," they will be included here for historical reasons. In the first one,[41] which is in fact based on a patent by Kvam,[43] it is shown that trifluoromethanesulfonamide **26** and structurally related perfluoroalkyl-/perchloroalkyl-sulfonamides of type **27**, are not only very potent *in vitro* CAIs against isozymes I–IV, but they also lower IOP in normotensive and glaucomatous rabbits as efficiently as MK-927 (**18**) or dorzolamide (**8**) when administered topically in 2% water solution (obviously, no ring systems are present in these perhaloalkylsulfonamides). Three facts are remarkable about this finding: (1) aliphatic sulfonamides were generally considered ineffective CAIs,[21] but these reports[40,41] definitively confuted this thesis; (2) aliphatic sulfonamides, such as **26**, also acted as very effective CA III inhibitors, although this isozyme was previously considered to be "sulfonamide-resistant," being generally inhibited only by millimolar concentrations of aromatic/heterocyclic sulfonamides of type **4–8** (Table II);[15,21] (3) it should be possible to design effective antiglaucoma drugs with a very simple chemical structure. In fact, **26** is only slightly more complicated (from the chemical point of view) than acetic acid. Still, this simple sulfonamide could not be developed for clinical use due to its chemical instability. Compound **26** is hydrolyzed *in vivo* to triflic acid (one of the most potent acids known), and this precluded with further investigations in this direction.[41]

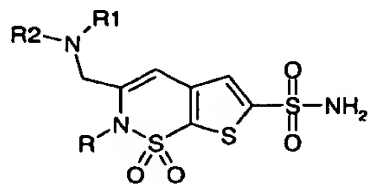
The second approach of Maren's group[42] mentioned above dealt with sulfamates of type **28**. In this case, the change of the classical aromatic/heterocyclic sulfonamide zinc binding function was even more drastic as compared to the aliphatic sulfonamides mentioned above: compounds **28** possess the general formula $R\text{-}O(CH_2)_nO\text{-}SO_2NH_2$, and they probably coordinate to the Zn(II) ion of the enzyme similarly to the aromatic sulfonamides, through the sulfamate nitrogen atom. Thus, these studies were extremely important because they enlarged the classes of available CAIs, from the generally accepted aromatic/heterocyclic derivatives, such as to include also the aliphatic sulfonamides, as well as compounds with a modified sulfonamide moiety, among which the sulfamates were the first example. Some compounds of type **28** showed affinity to CA II in the 10^{-8} M range, as well as very good water solubility as hydrochloride salts, and were formulated as eye drops.[42] They also showed moderate IOP lowering in normotensive rabbits, but were not further evaluated for clinical development, probably due to their modest efficacy as compared to dorzolamide and its precursor, MK-927.

The second clinically used topical CAI brinzolamide (**9**) was developed by Alcon Laboratories, probably by using dorzolamide (**8**) as lead.[44] In fact, the two compounds are structurally similar, with brinzolamide belonging to the 2-substituted-2*H*-thieno[3,2-*e*]-1,2-thiazine-6-sulfonamide class. The main difference with the Merck derivatives is that the Alcon researchers generally did not publish in scientific journals their chemistry, but only patented these compounds.[44] Only recently some brinzolamide congeners are described in some detail in a published article.[45]



28: n = 2,3

R = m- or p-



29: R = Me, Et, Pr, allyl, MeO-$(CH_2)_3$, etc.

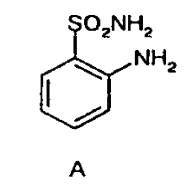
NR1R2= 4-morpholinyl; $N(CH_2CH_2OMe)_2$, etc.

Similar to the thienothiopyran sulfonamides **18** and **8** (or the thienothiazine variant **19**) developed by Merck, the 2-substituted-2*H*-thieno[3,2-*e*]-1,2-thiazine-6-sulfonamides to which brinzolamide (**9**) belongs, act as low nanomolar inhibitors for hCA II, being slightly less effective as hCA IV inhibitors. On the other hand, the brinzolamide type compounds are less water soluble as compared to dorzolamide, and thus, they are formulated as suspensions for topical administration. Another important characteristic is that they are more effective than dorzolamide, and produce less eye stinging and burning. A defect is that they provoke much more blurred vision after administration, obviously due to the suspension nature of the formulation.[2,29,30]

A common problem of both dorzolamide and brinzolamide is that they contain chiral centers, and the preparation of the pure enantiomer ((4*S*,6*S*) in the case of dorzolamide and (4*R*) in the case of brinzolamide, respectively is rather expensive. Thus, recently, the Alcon group reported some brinzolamide-like compounds of type **29**,[45] which do not contain chiral centers. These derivatives principally differ from brinzolamide by the absence of the 4-substituent (which induced the chirality), by a rather large number of substituents in the 2-position of the heterocyclic ring (the R group of formula **29**), as well as by the presence of an additional double bond in the six-membered heterocycle annulated to the thiophene nucleus, which represents an innovative feature as compared to the previously topically active sulfonamides prepared by the ring approach. These compounds were effective nanomolar inhibitors against hCA II and hCA IV, and showed good IOP lowering (of 20–30%) properties in naturally hypertensive Dutch-belted rabbits, after administration as suspensions (except for two derivatives, which were soluble enough to be administered in solution at pH 5.5).[45] It is not clear at this moment whether such compounds may substitute brinzolamide in the near future, as a second generation topically acting sulfonamides.

C. The "Tail" Approach

This approach has been developed in our laboratory [46–60] and consists in using well-known aromatic/heterocyclic sulfonamide scaffolds (of type A–Y) to which tails that will induce water solubility are attached at the amino, hydroxy, imino, or hydrazino moieties contained in the precursor sulfonamides A–Y.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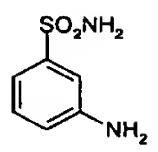
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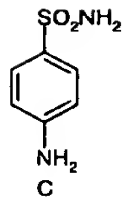
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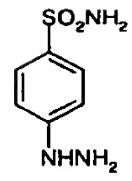
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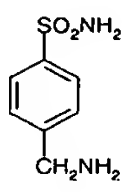
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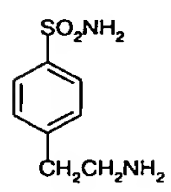
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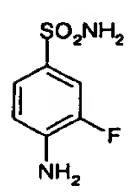
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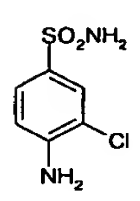
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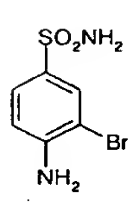
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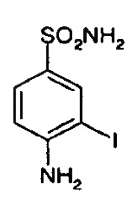
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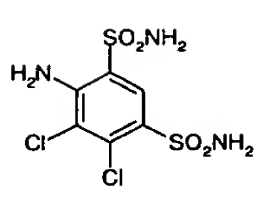
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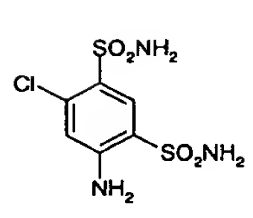
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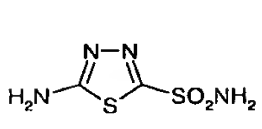
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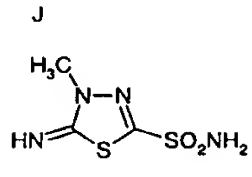
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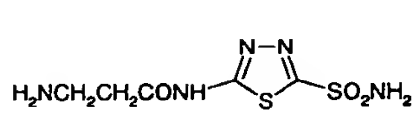
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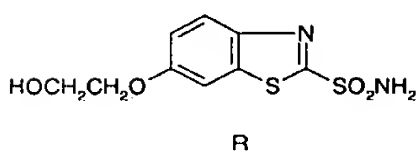
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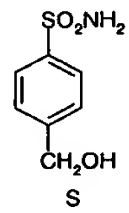
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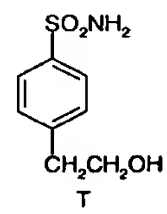
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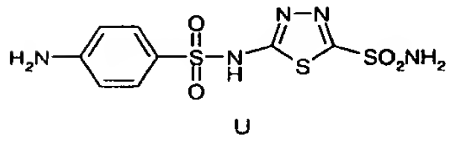
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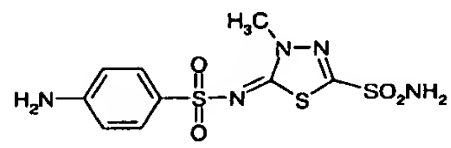
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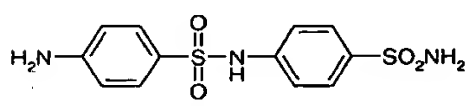
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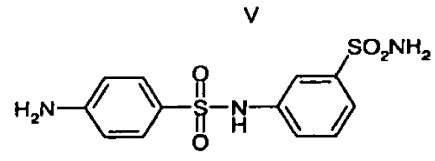
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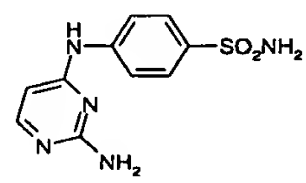
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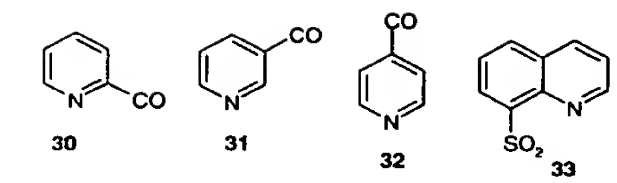
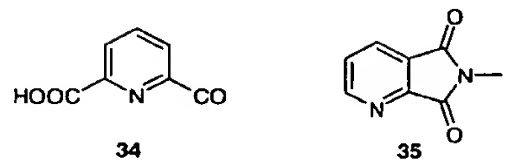
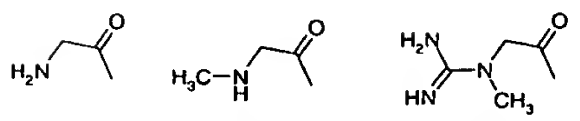


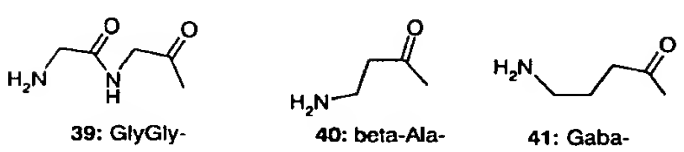
30 31 32 33



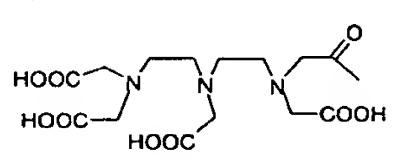
34 35



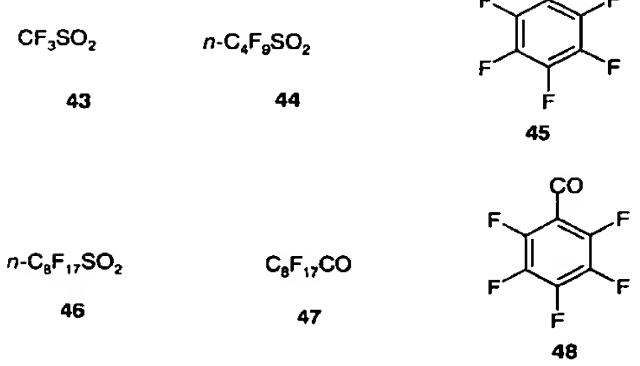
36: Gly- 37: Sar- 38: Crt-



39: GlyGly- 40: beta-Ala- 41: Gaba-



42: dtpa-



43 44 45

46 47 48

The starting sulfonamides derivatized by this simple approach included 2-, 3-, or 4-aminobenzenesulfonamides, 4-(ω-aminoalkyl)-benzenesulfonamides/thiadiazole-sulfonamides, 3-halogeno-substituted-sulfanilamides, 1,3-benzene-disulfonamides, 1,3,4-thiadiazole-2-sulfonamides, benzothiazole-2-sulfonamides, as well as sulfanilyl-substituted aromatic/heterocyclic sulfonamides (structures U-X) among others, and were chosen in such a way as to prove that this is a general approach.[46–60] Tails that were introduced in the molecules of such new CAIs contained either moieties protonable at endocyclic nitrogen atoms (such as pyridine- or quinoline rings **30–35**), or at amino groups belonging to amino acids and some of their derivatives (such as glycine, β-alanine, GABA, sarcosine, creatine, diethylenetriaminopentaacetic acid (dtpa), or glycyl-glycine moieties of types **36–42**), as well as perfluoroalkyl/aryl moieties (which are not protonable at pH values in the neutral range, of types **43–48**). The water solubility of the first type of such derivatives is assured by formation of salts with hydrochloric, trifluoroacetic or triflic acid, or by formation of sodium salts, for the derivatives possessing carboxylic acid moieties (tails of types **34** or **41**). The perfluoroderivatives (incorporating tails **43–48**) constitute a special case that will be discussed later. The tail was generally attached to the sulfonamide scaffold either as an amide (CONH), ester (COOR), sulfonamide (SO_2NH) or imide moiety (in one case, the tail **35**, which was in fact obtained by reaction of pyridine-2,3-dicarboxylic acid anhydride with amino-sulfonamides, leading to phthalimide-like compounds.[46]) Thus, a sulfonamide CAI obtained by the tail approach is generally described by a

figure (corresponding to the tail moiety) and a letter (corresponding to the sulfonamide scaffold to which the tail has been attached, through a carboxamide, ester or secondary sulfonamide bond). For instance, **30C** should be the isonicotinoylamido derivative of sulfanilamide, **36M** the glycinamido derivative of 5-amino-1,3,4-thiadiazole-2-sulfonamide, etc.[46–60]

The main advantages of this over the ring approach are that the first is much more simple than the second, and it allows a parallel type synthesis easily, so that a large number of different derivatives may be prepared, and their physico-chemical properties modulated in such a way as to assure the desired pharmacological properties. By choosing different tails, it is possible to attain the much desired water solubility of these CAIs (as salts of acids or bases) at pH values in the neighborhood of neutrality, avoiding thus the irritation problems observed with the strongly acidic dorzolamide solutions. In fact, many of the protonatable moieties of tails **30–42** possess pKa values in the range of 6–7, which is quite advantageous for the solubility of these derivatives at pH values in the almost neutral range. Furthermore, some of these derivatives also contain carboxylic acid moieties in addition to the protonatable amino moieties, which allows the obtaining of water solutions of their sodium salts, with pH values of 7.0–7.5. Such solutions were never irritating for the eye of experimental animals.[46–60]

Sulfonamides obtained by incorporating pyridine-carboxamido- or quinoline-sulfonamido tails (of types **30–35**) into the scaffolds A–Y were in many cases nanomolar inhibitors of isozymes hCA II and bCA IV,[46–52] possessed good water solubility in the range of 1.5–2%, and the pH of their solutions used for *in vivo* experiments was in the range of 6.5–7.5. Many of these compounds were very effective IOP lowering agents in normotensive and glaucomatous albino rabbits, with potencies 2–3 times superior to dorzolamide.[46–52]

New derivatives prepared by the tail approach, incorporating amino acyl-, oligopeptidyl-, or polyaminopolycarboxyl-moieties (of types **36–42**) were again potent inhibitors of isozymes CA I, CA II, and CA IV, and could be formulated in water solutions in the concentration range of 2–2.5%, at almost neutral pH values (pH 6–7).[53–55,59]

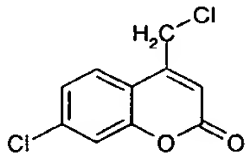
A special case of this approach was constituted by the perfluoroalkyl-/aryl-containing derivatives, which incorporated tails **43–48**.[58] Such compounds are reminiscent of the trifluoromethanesulfonamide structure **26** (and the related derivatives **27**) investigated by Maren's group.[41] Obviously, such compounds (for example **45C** or **45M**) cannot form water soluble salts with acids or bases at neutral pH. To our greatest surprise, this class of derivatives was shown to possess an unexpectedly high water solubility (in the range of 1.5–2%!), balanced by a significant lipid solubility (due to the presence of the perfluoroalkyl-/aryl moieties).[58] Correlated with their very good CA inhibitory properties against isozymes CA I, CA II, and CA IV (in the nanomolar range), such derivatives possess optimal properties to act as efficient IOP lowering agents: they can be formulated as water solutions at neutral pH; they possess a very good penetrability through the cornea (due to the good lipophilic character), arriving thus at the ciliary processes enzyme; their duration of action is much prolonged as compared to that of dorzolamide. Indeed, compounds such as **45M** or **44N** among others showed a very strong IOP lowering effect both in normotensive, as well as glaucomatous rabbits, and this IOP lowering effect for 5–6 hr (in the case of dorzolamide, pressure returns to basal levels after a much shorter period).[58]

It appeared obvious from these studies that the tail incorporated into the molecules of such CAIs is important for at least three critical properties of the antiglaucoma agent: (i) to assure the water solubility of the drug, in order to formulate it as a solution for ophthalmologic use. By the tail approach it is possible to formulate topically acting antiglaucoma sulfonamides as 1.5–2% solutions, at pH values in the neutral pH range (pH values of 6.5–7.5, either as a salts of a strong acid, either as salts of a strong base);[46–58] (ii) to assure optimal penetration of the drug through the cornea, in order to inhibit the ciliary body enzymes (CA II and CA IV), and this is mainly realized if the inhibitors possess a modest, but not insignificant lipophilicity. In fact, highly lipophilic sulfonamides are readily washed away from the eye due to the blood circulation (where high amounts of CA I and CA II are present),

whereas too hydrophilic compounds do not have the chance to penetrate through the membranes, in order to arrive at the ciliary process enzyme;[46–60] (iii) the tail should assure high affinity for the enzyme (mainly the isozyme II), with nanomolar inhibitors as the best candidates for clinical development as antiglaucoma drugs.

The tail approach is a general one, as shown by the multitude of highly active topically effective antiglaucoma sulfonamides reported in the last years.[45–60] Several variants of the main approach exposed above have also been designed and will be exemplified in the following.

Thus, sulfonamides of type A–Y possessing free amino, imino or hydrazino moieties were reacted with 7-chloro-4-chloromethylcoumarin **49**, affording a series of secondary amines possessing *N*-[(7-chloro-4-coumarinyl)-methyl]- moieties in their molecules, which showed effective inhibition of three carbonic anhydrase isozymes (CA I, II, and IV) and were water soluble as hydrochloride salts.[56] Some of these derivatives also showed IOP lowering properties in the normotensive rabbits after topical application as 2% water solutions, but their efficiency was not as good as that of the previously mentioned derivatives.[56] In another paper, some of the above mentioned sulfonamides (A–Y) were derivatized by means of furan-, pyrrole-, and thiophene-carboxamido moieties of type **50**.[57] The new derivatives obtained in this way were not water soluble and they were formulated as suspensions for the *in vivo* experiments, similarly to brinzolamide (**9**). The compounds incorporating furan- and pyrrole-carboxamido moieties (but not the corresponding thiophene-substituted derivatives), showed effective and long-lasting IOP lowering both in normotensive, as well as glaucomatous animals, with potencies superior to dorzolamide and brinzolamide, and this was explained by the insufficient lipophilicity of the latter derivatives as compared to the structurally related furan- and pyrrole-carboxamido containing compounds, since all of them showed nanomolar affinity for hCA II and bCA IV.[57]



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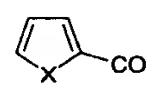
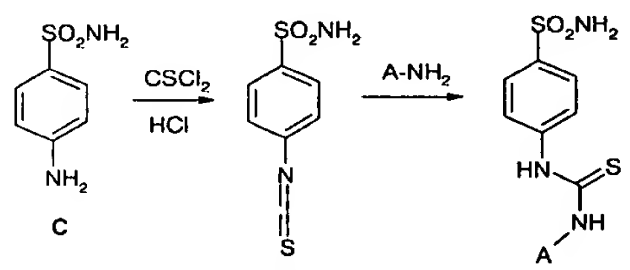
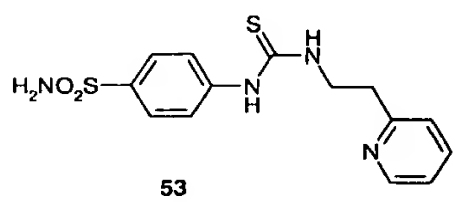


50 **A**: X = O
B: X = NH
C: X = S



C **51** **52**



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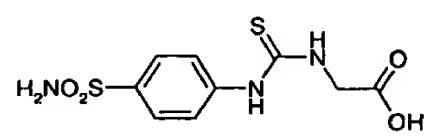

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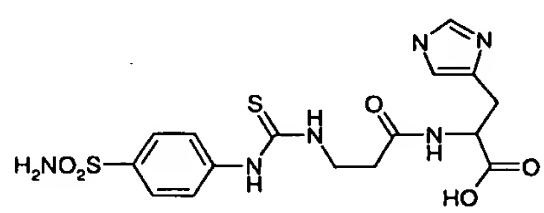
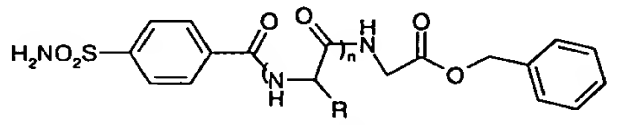
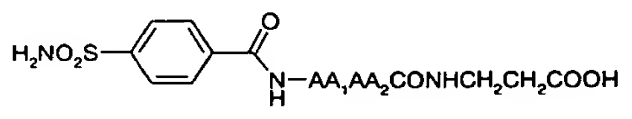


55

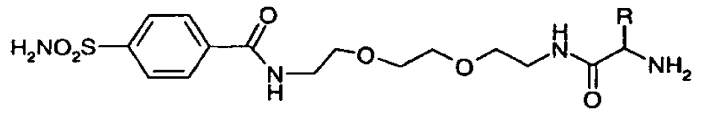
A new type of chemistry was recently reported by Casini et al.,[60] who prepared 4-isothiocyanato-benzenesulfonamide **51** by treating sulfanilamide C with thiophosgene. This compound was then used for the preparation of a large number of thioureas **52**, by reaction with amines (such as histamine, phenethylamine, etc.), amino acids (amino-benzoic acids, all the twenty natural amino acids, as well as many non-natural derivatives), as well as di-, tri-, and tetrapeptides.[60] Some of these compounds (such as **53**) showed very good water solubility as hydrochloride or TFA salts, whereas the amino acid or oligopeptide derivatives (such as **54** and **55** among others) were water soluble as sodium salts. Many of these derivatives were very potent CA inhibitors (against the isozymes I, II, and IV) and effectively reduced IOP in both normotensive/glaucomatous rabbits.[60] Similarly to the perfluoroalkyl/aryl sulfonamides discussed earlier, these compounds also showed a prolonged duration of action as IOP lowering agents.



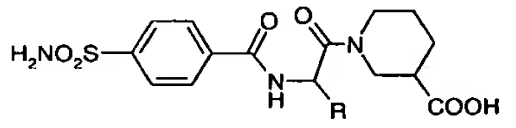
56: R = H, Ph; $PhCH_2$; n = 2-4



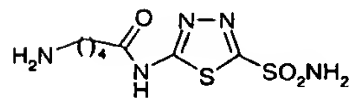
57: AA_1 = AA_2 = L-Leu; D-Leu; L-Thr; D-Thr; L-Ser; D-Ser; Gly



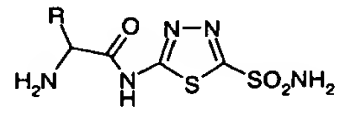
58: Gly; Leu; Ser; Lys; Glu; Phe derivatives



59: R = *i*-Pr-CH_2; $HOOCCH_2$; $H_2NCOCH_2CH_2$



60



61: R = H
62: R = Me
63: R = $PhCH_2$

It must also be mentioned that a similar approach to the "tail" one described here was reported by Whitesides' group who studied the increased affinity to the enzyme of compounds possessing secondary binding sites adjacent to the sulfonamide one.[61–64] This strategy exploited hydrophobic interactions between hydrophobic patches at the entrance of the hCA II active and similar moieties of the inhibitor molecule, and led to very effective CAIs of types **56–58**, which were generally obtained by derivatizing 4-carboxy-benzenesulfonamide with aminoacyl, oligopeptidyl or ethyl-eneglycol moieties. These compounds were generally very effective CA I and CA II inhibitors, but

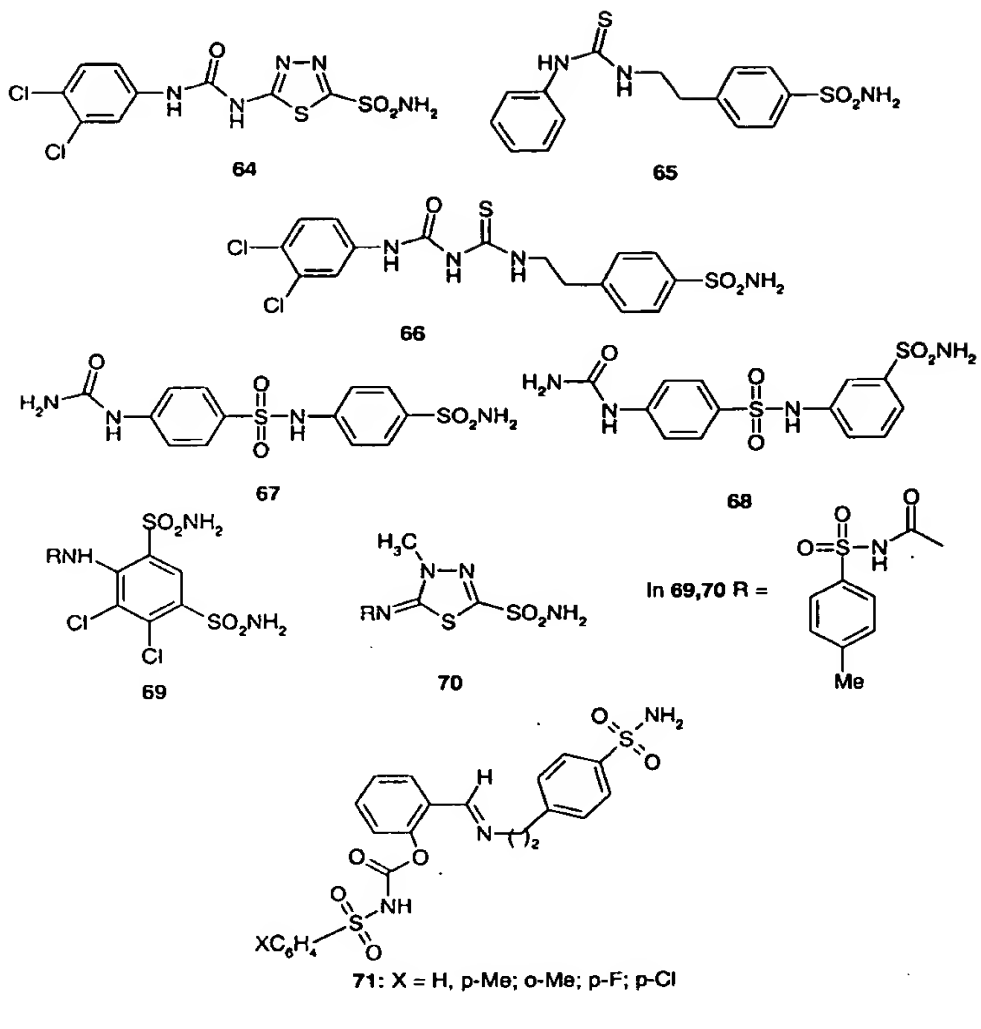
were not investigated—as far as we know—for their antiglaucoma properties. Similar derivatives were also reported by Burbaum et al.[65] (derivatives of type **59**), Antonarolli et al.[66] (compound **60**) and Jayaweera et al.[67]—derivatives **61–63**. Except for **60** (with weak IOP lowering properties[66]) and **61**, **62** (stated to be active, but no quantitative data provided in Ref.[67]), these compounds were scarcely investigated for their putative topical antiglaucoma properties, and they were included here more for historical reasons, as precursors of the compounds obtained by the real "tail" strategy presented above.

D. Isozyme-Specific Inhibitors

Although many sulfonamide CAIs possess high affinity for the major isozymes considered to play important physiological functions (such as CA II, CA IV, and CA V),[2,7,21,26,27] the critical challenge for the design of novel pharmacological agents from this class is constituted by the lack of specificity of such compounds towards the different isozymes.[2,26] Among the 14 isozymes described up to now, just CA II and CA IV have very similar affinities to the sulfonamide inhibitors, although, as mentioned above, small differences between them exist, with CA II showing a slightly larger affinity than CA IV for the largest majority of such compounds (Table II).[2] This fact, as well as the physiological importance of these two isozymes, prompted much research in many laboratories in order to find compounds which might discriminate between CA II and CA IV. The same is true for finding inhibitors with higher affinity to CA I, as compared to the sulfonamide-avide isozyme II, mainly because the physiological function of CA I is still a mystery, although this protein is very abundant in many mammals, including humans.[2,26] Things are relatively simpler for CA III, which is a "sulfonamide-resistant" isozyme, although it is inhibited well by small compounds such as $CF_3SO_2NH_2$.[41] Mention should be made that very few data are available regarding the inhibition of isozymes other than CA I–CA IV at the moment. Some progress has been registered recently in the design of compounds with some selectivity towards CA I and CA IV, and these data will be reviewed in this section.

E. Isozyme I

The main difference in the active site architecture of isozymes CA I and CA II regards the presence of a higher number of histidine residues in the first isozyme. Thus, in addition to the zinc ligands (His 94, His 96, and His 119), as shown in the introductory section, His 64 plays an important role in catalysis. This is the only other histidine residue present in the active site of CA II, whereas in CA I there are three additional such residues, His 67, His 200, and His 243.[10] Another important difference between the two isozymes is that CA II contains a histidine cluster, consisting of residues: His 64, His 4 (these two residues possess a flexible conformation in the crystal structure,[7,10]) His 3, His 10, His 15, and His 17 (prolonging from the middle of the active site to the rim of the cavity, and protruding on the surface of the protein,[10]) which is absent in CA I. These two isozymes also possess a different affinity for the two main classes of inhibitors: CA I has larger affinity than CA II for anions (such as cyanide, thiocyanate, cyanate, halides, etc), whereas CA II has generally a higher affinity for sulfonamides as compared to CA I (see also Table II).[2,13,14] As a consequence, it is relatively difficult to obtain sulfonamide inhibitors with higher affinity for CA I than for CA II, although the two isozymes possess significant differences in the active site architecture. The first such compounds were only recently reported by this group,[68–71] and were discovered serendipitously, by screening of a large number of sulfonamides possessing different structural motifs in their molecules. Remarkably, all the compounds possessing higher affinity for CA I as compared to CA II (and CA IV), of types **64–71**, contain ureido or thioureido moieties in their molecules. Their inhibition data against the three isozymes mentioned above are shown in Table III.



Such isozyme I avid inhibitors belong both to the aromatic sulfonamide class (such as **65–69** and **71**), as well as to the heterocyclic sulfonamide class (**64, 70**), whereas the ureido/thioureido moieties present in their molecules may be unsubstituted (such as in **67** and **68**) or substituted with bulkier groups (3,4-dichlorophenyl; phenyl; substituted-phenylsulfonyl, etc). It must also be mentioned that

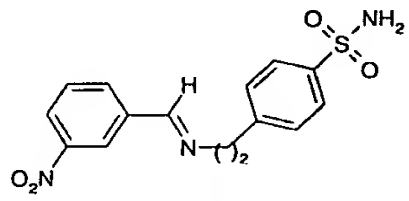
Table III. Inhibition of Isozymes I, II, and IV by Compounds **64–76** Showing Selectivity Towards One of These Isozymes

	K_I (nM)		
Inhibitor	*hCA I*	*hCA II*	*bCA IV*
64	3	6	8
65	50	53	70
66	7	10	24
67	3	8	20
68	4	10	25
69	8	12	14
70	4	5	11
71a (X = 4-Me)	40	110	120
71b (X = 4-Cl)	60	100	160
72	1,100	150	140
73	200	20	10
74	200	10	8
75	620	12	10
76	180	15	12

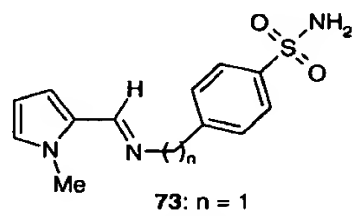
compounds **71** containing arylsulfonylcarbamate moieties instead of the arylsulfonylureido ones were investigated in more details.[68–71] These compounds also inhibit significantly isozymes II and IV, and thus, are more isozyme-I selective, but represent anyhow an important step towards the generation of isozyme specific CAIs. It must also be noted that dorzolamide (**8**) has a very low affinity for hCA I, but its de-ethylated metabolite is a very potent inhibitor of this isozyme.[2,29]

F. Isozyme IV

Isozyme CA IV contains only one histidine residue within its active site, His 64, which as in hCA II, plays a critical role in catalysis, as proton shuttle residue between the active site and the environment.[7] The most characteristic feature of the active site of this isozyme is related to the presence of four cysteine residues, which form two disulfide bonds, situated at the entrance within the cavity (Cys 6–Cys 11G, and Cys 23–Cys 203, respectively).[7] These residues occupy practically the same region of the active site as the histidine cluster in hCA II,[10] and it was hypothesized that this might be the most relevant aspect explaining the difference in affinity for sulfonamide inhibitors of these two isozymes.[72] Even so, similarly to CA I, the first compounds with some specificity for CA IV, of type **72–76**, were again discovered serendipitously, and they all belong to the same class of Schiff bases of aromatic/heterocyclic sulfonamides.[73–76]

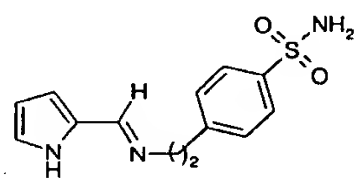


72

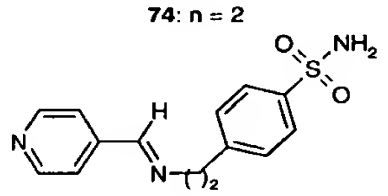


73: n = 1

74: n = 2



75



76

Only Schiff bases of aromatic sulfonamides were investigated in some detail, and it was shown that best CA IV inhibition patterns are connected with the presence of heterocyclic moieties (in the original aldehyde used for the preparation of the Schiff base), or aromatic moieties substituted with electron attracting groups, such as the nitro one.[73–76] Such compounds also appreciably inhibited CA II, and to a smaller extent CA I (Table III).[73–76]

G. Isozyme III

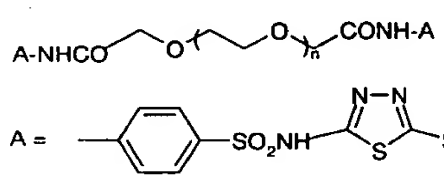
Although the structure of this isozyme is relatively similar to that of hCA II, CA III has a CO_2 hydration activity of about 0.3% that of hCA II, as it does not possess a His residue in position 64, but a Lys residue, which is much less effective as a proton shuttle.[77] Furthermore, position 198 in CA III is occupied by a Phe, possessing a very bulky side chain, whereas the water bound to Zn(II) has a pK_a around 5.5.[77] All these particularities may explain the low catalytic activity of CA III, as well as its insensitivity to sulfonamide inhibitors, which do not have enough space to bind in the neighborhood of the Zn(II) ion, principally due to the steric impairment of Phe 198.[77] In fact, only the very small sulfonamide $CF_3SO_2NH_2$ acts as an efficient CA III inhibitor, with an inhibition constant of 0.9 μM

(but this compound is a nanomolar inhibitor of CA I, II, and IV on the other hand).[41] Other sulfonamides (such as acetazolamide, methazolamide, etc) inhibit CA III, with inhibition constants in the millimolar range.[41]

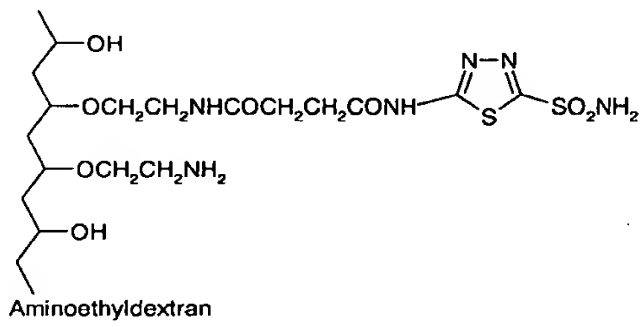
II. Positively Charged Sulfonamides as Selective Inhibitors of Membrane Associated CAs

At least four CA isozymes (CA IV, CA IX, CA XII, and CA XIV) are associated to cell membranes, with the enzyme active site generally oriented extracellularly.[1,2] Some of these isozymes were shown to play pivotal physiological roles (such as for example CA IV in the eye, lungs and kidneys, CA IX in the gastric mucosa and many tumor cells),[1,2] whereas the function of other such isozymes (CA XII, CA XIV) is for the moment less well understood. Due to the extracellular location of these isozymes, it would be possible to design membrane-impermeant CAIs, which in this way would become specific inhibitors for the membrane-associated CAs. This possibility has been fully explored in this laboratory, by designing positively charged sulfonamides,[72,78–82] whereas an alternative approach consisted in designing polymeric (high molecular weight) inhibitors, but such compounds were not very useful *in vivo* due to the usual problems connected with polymers (i.e., allergic reactions, problems of bioavailability, etc).[83–86]

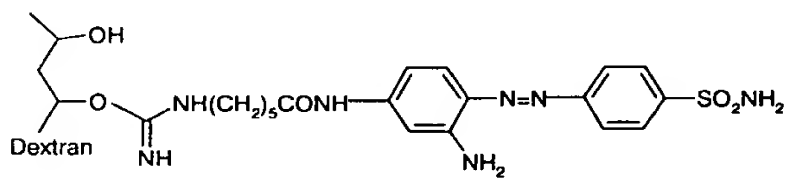
Thus, the first approach for inducing membrane-impermeability to CAIs from the historical point of view was that of attaching aromatic/heterocyclic sulfonamides to polymers, such as polyethyleneglycole,[83] aminoethyldextran,[84,85] or dextran.[86] Compounds such as **77–79**, possessing molecular weights in the range of 3.5–99 kDa, prepared in this way, showed indeed membrane-



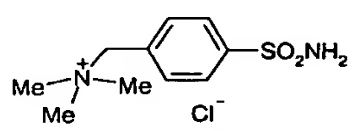
77: M_r = 3.5 kDa



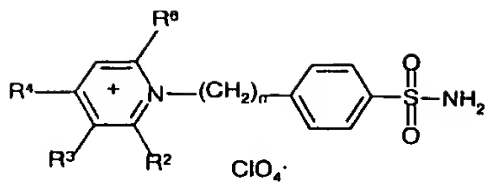
78: M_r = 6.7 - 99 kDa



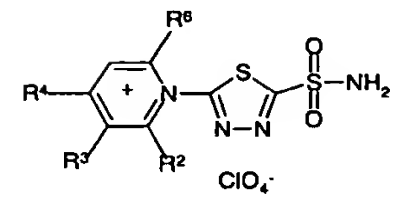
79: Mr = 5, 100 and 1000 kDa



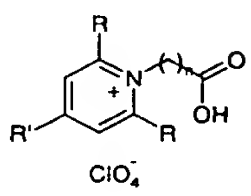
80: QAS



81: n = 0
82: n = 1
83: n = 2

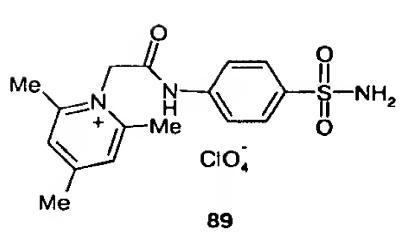


84

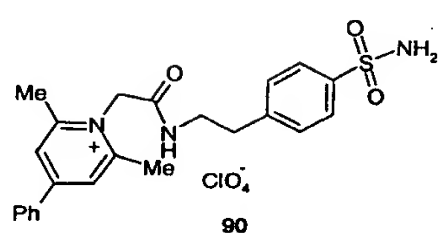


n = 1, 2

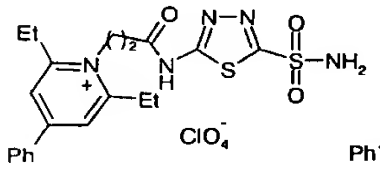
85: R = R' = Me
86: R = Me; R' = Ph
87: R = Et; R' = Ph
88: R = i-Pr; R' = Ph



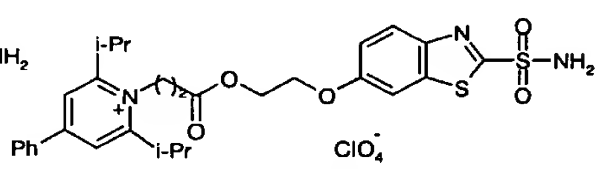
89



90



91



92

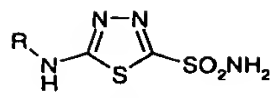
impermeability due to their high molecular weights, and selectively inhibited *in vivo* only CA IV and not the cytosolic isozymes (primarily CA II), being used in several renal and pulmonary physiological studies.[83–86] Due to their macromolecular nature, such inhibitors could not be developed as drugs/diagnostic tools, since *in vivo* they induced potent allergic reactions. A second approach for achieving membrane-impermeability is that of using highly polar, salt-like compounds. Only one such sulfonamide has been used in physiological studies, QAS (quaternary ammonium sulfanilamide) **80**, which has been reported by Henry[87] to inhibit only extracellular CA-s in a variety of arthropods (such as the crab *Callinectes sapidus*) and fish. The main drawback of QAS is its high toxicity in higher vertebrates.[21]

Thus, a program of developing cationic sulfonamides has been initiated in our laboratory,[78,79] using QAS (**80**) as lead molecule (which is also a relatively weak CAI, with micromolar affinity for CA II.[21,79]) The first such compounds, of types **81–84**, were prepared by reaction of aromatic/heterocyclic sulfonamides containing free NH_2 groups with pyrylium salts, affording pyridinium derivatives.[72,78–80] These compounds were moderately active CA II and CA IV inhibitors, with affinities in the 10^{-6}–10^{-7} M range. By using QSAR data from this laboratory,[79,88–91] it has been shown that increased CA II and CA IV inhibitory properties of aromatic/heterocyclic sulfonamides are connected with the presence of elongated inhibitor molecules (on the axis passing through the Zn(II) of the enzyme, the sulfonamide nitrogen atom and the long axis of the inhibitor molecule itself). In consequence, such "elongated" molecules have been designed[81,82] by reacting pyrylium salts with

amino acids (such as glycine or β-alanine), and coupling of the pyridinium derivatives **85–88** with the aromatic/heterocyclic sulfonamides possessing free amino, hydroxy, imino or hydroxyl moieties of types A–Y (see Section 3.1.2). The inhibitors obtained in this way, such as for instance **89–92**, showed nanomolar affinities both for CA II, as well as CA IV, and more importantly, they were unable to cross the plasma membranes *in vivo*.[81,82] In two model systems (human red cells, and perfusion experiments in rats, respectively), this new class of potent, positively charged CAIs, was able to discriminate for the membrane-bound *versus* the cytosolic isozymes, selectively inhibiting only CA IV.[81,82] Such data are important both for the specific *in vivo* inhibition of membrane-associated isozymes, but also for the eventual development of some novel anticancer therapies, since it has been shown that some tumor cells predominantly express only some membrane-associated CA isozymes, such as CA IX and CA XII.[92,93]

1. Antitumor Sulfonamides

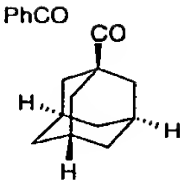
There are many connections between CA and cancer.[1,2,92–94] As mentioned above, some CA isozymes are predominantly found in cancer cells and are lacking from their normal counterparts.[92–94] Teicher et al.[95] reported that acetazolamide (**4**), one of the best-studied, potent inhibitors of several CA isozymes (CA II, CA IV, CA V, and CA VII among others, see Table II) used clinically, might also function as a modulator in anticancer therapies in combination with different cytotoxic agents, such as alkylating agents, nucleoside analogs, platinum derivatives, etc. It was hypothesized that the anticancer effects of acetazolamide (alone or in combination with such drugs) might be due to the acidification of the intratumoral environment ensued after CA inhibition, although other mechanisms of action of this drug were not excluded.[95] Our and Puscas's groups[96] also showed that by modulating the CA activity (by means of inhibitors or activators[97] of this enzyme) the pH of the tumor environment can be changed, which may favorably influence the anticancer effect of the drug *per se* (i.e., the sulfonamide CA inhibitor) or that of another anticancer agent used concomitantly with the CA inhibitor/activator. Chegwidden and Spencer[98] then showed that two other potent, clinically used sulfonamide CAIs, methazolamide (**5**), and ethoxzolamide (**6**) (in concentrations of 0.4–1 mM for **5** and around 10 μM for **6**) inhibited *in vitro* in cell cultures, the growth of human lymphoma cells, showing that this is probably due to a reduced provision of bicarbonate for nucleotide synthesis (HCO_3^- is the substrate of carbamoyl phosphate synthetase II) as a consequence of CA inhibition. Since different isozymes such as CA I, II, and IV were recently shown to be present and probably involved in other types of proliferative conditions, such as von Hippel-Lindau tumors,[94] progressive polycystic kidney disease,[99] acinar-ductal carcinomas of the pancreas or autoimmune/idiopathic chronic pancreatitis,[100] it appeared of great interest to further explore the connections between CAs and tumors. The development of CAIs that also show potent tumor cell growth inhibitory properties was recently reported by our group.[101–104] Such compounds were in fact discovered in a large screening program of sulfonamide CAIs, in collaboration with NIH.[101–104] Several hundred aromatic/heterocyclic sulfonamides were assessed *in vitro* as potential inhibitors of growth of a multitude of tumor cell lines, such as leukemia, non-small cell lung cancer, ovarian, melanoma, colon, CNS, renal, prostate, and breast cancers. The active compounds (most of them nanomolar inhibitors of CA II and CA IV), of types **93–103**, belong both to the aromatic, as well as to the heterocyclic sulfonamide classes, and showed GI_{50} values (molarity of inhibitor producing a 50% inhibition of tumor cell growth after 48 hr exposure to the drug) in the micromolar range.[103,104] Better antitumor compounds were then developed by an original strategy, and they incorporated in their molecules *N,N*-dialkylthiocarbonylsulfenylamino moieties.[101,102] Thus, aromatic/heterocyclic sulfonamides possessing free amino, imino or hydrazino groups of types A–Q, U–Y were transformed into the corresponding *N*-morpholyl-thiocarbonylsulfenyl,[101] or *N,N*-dimethyl/diethyl-thiocarbonylsulfenylamino-derivatives **104–106**,[102] by reaction with dithiocarbamates in the presence of oxidizing agents (NaClO or iodine).



93-99

93: R = PhCO

94: R =

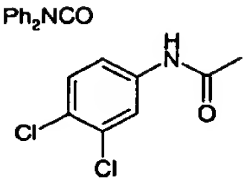


95: R = O_2N—S—

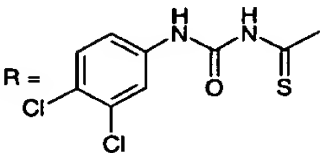
96: R = O_2N—SO_2—

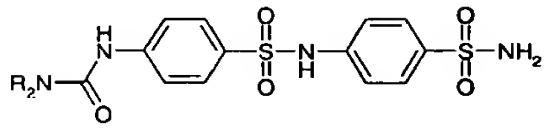
97: R = Ph_2NCO

98: R =

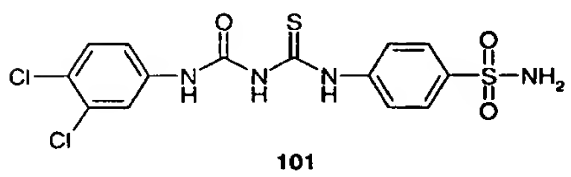


99: R =

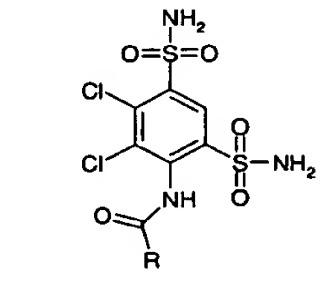




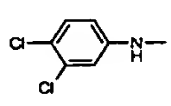
100: R = H; 5: R = Ph



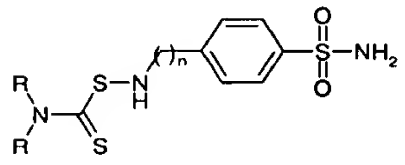
101



102: R =



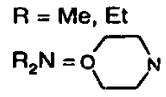
103: R = Ph_2N-



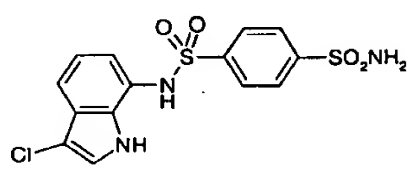
104: n = 0
105: n = 1
106: n = 2

R = Me, Et

R_2N =



K_I = 10 - 35 nM (hCA II)
GI_{50} = 10 - 65 nM
(different tumor cell lines)



107: E7070

Sulfonamides of type **104–106** showed nanomolar affinity for CA II and CA IV, but what is more important, some of them inhibited the growth of several tumor cell lines at concentrations as low as 10 nM,[101,102] showing thus a highly increased antitumor efficacy as compared to the classical CAIs (acetazolamide, methazolamide) or the previously mentioned compounds **93–103**. It must be also mentioned that an antitumor sulfonamide, E7070 (**107**), recently reported by Owa's group,[105] is in phase II clinical trials in Europe as a novel anticancer agent.[24] As far as we know, this compound has not been assayed as a CA I, by possessing the unsubstituted sulfonamide moiety we predict that it behaves as a strong inhibitor.

J. Sulfonamides With Modified Moieties and Hydroxamates With CA Inhibitory Properties

Krebs[25] reported in 1948 that substitution of the sulfonamido moiety in compounds of type $ArSO_2NHR$ drastically reduce the CA inhibitory properties as compared to the corresponding derivatives possessing primary sulfonamido groups, $ArSO_2NH_2$. As a consequence, other zinc-binding functions except for the SO_2NH_2 one have rarely been taken into consideration in the design of CAIs, although many other zinc enzymes are inhibited by a multitude of derivatives possessing an entire range of zinc binding functions, such as thiols, phosphonates, carboxylates, hydroxamates, etc.[18,106] Only recently several detailed studies regarding the possible modifications of the sulfonamido moiety, compatible with the retention of strong binding to the enzyme, have been reported.[14,107] Compounds of type **108–110** (Table IV) were studied kinetically, for inhibition of reactions catalyzed by CA I and II (CO_2 hydration and ester hydrolysis), but their binding to the enzyme has also been monitored spectroscopically, by studying the electronic and ^{1}H-NMR spectra of adducts of such inhibitors with Co(II)-CA II.[14]

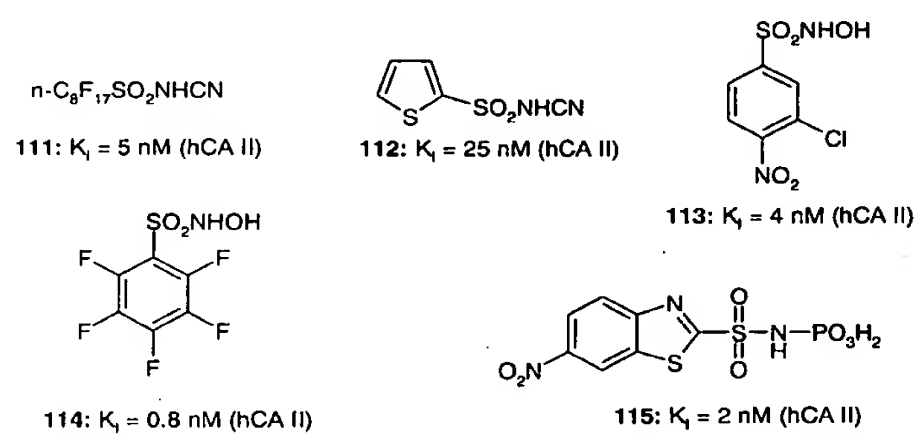
Thus, for a series of derivatives with modified sulfonamido moieties **108–110** (Table IV), it has been observed that the presence of bulky substituents at the sulfonamido moiety (such as phenylhydrazino, ureido, thioureido, guanidino, etc) led to compounds with weak inhibitory properties, whereas moieties present in inorganic anion CAIs (such as NO, NCS, N_3) or compact moieties substituting the sulfonamide nitrogen (such as OH, NH_2, CN, halogeno) led to compounds with appreciable inhibitory properties.[14,107] For example, the *N*-hydroxy sulfonamide **108b**, the *N*-chloro-substituted derivatives **108j,k**, as well as the nitroso- and thiocyanato derivatives **108d,e** possessed the same affinity for the two investigated isozymes as the unsubstituted sulfonamide **108a**.

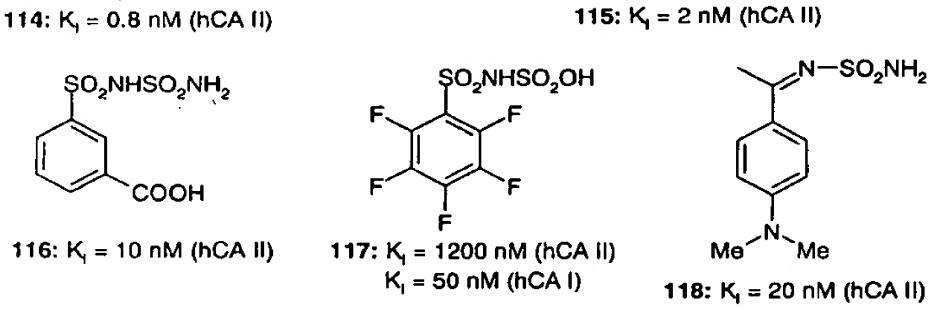
Table IV. Inhibition of hCA I and hCA II with Compounds Incorporating Modified Sulfonamide Moieties (**108a–t**), Sulfamide (**109**), and Sulfamic acid (**110**)

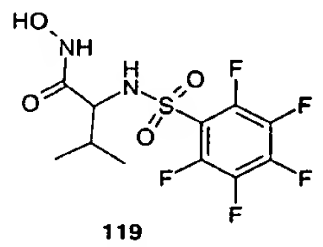
	4-Me-C_6-H_4SO_2-X **108a–t**	$H_2NSO_2NH_2$ **109**	$HOSO_2NH_2$ **110**
		K_I (*mM*)	
Inhibitor	*X*	*hCA I*	*hCA II*
108a	NH_2	50	11
108b	NHOH	41	9
108c	NHOMe	220	173
108d	NO	35	24
108e	NCS	30	18
108f	N3	27	45
108g	Imidazol-1-yl	160	34
108h	$NHNH_2$	70	53
108i	NHNHPh	> 1,000	120
108j	NHCl	19	2.1
108k	NCl_2	12	3.6
108m	NHCN	210	125
108n	$NHOCH_2COOH$	150	85
108p	OH	130	460
108q	SH	5	10
108r	$NHCONH_2$	> 1,000	460
108s	$NHCSNH_2$	> 1,000	410
108t	$NHC(NH)NH_2$	> 1,000	540
109		310	1,130
110		21	390

For the CO_2 hydration reaction, from Refs.[14,107].

Interestingly, the thiosulfonic acid (as sodium salt) **108q** was one of the best inhibitors in this series, in contrast to the sulfonate (as sodium salt) **108p**, which is a very weak inhibitor. Sulfamide **109** (the most simple compound containing a sulfonamido moiety) is an extremely weak inhibitor—but it binds to the Zn(II) ion, as showed by electronic spectroscopic studies on the Co(II)-substituted enzyme, whereas sulfamic acid **110** is a much stronger inhibitor. Furthermore, this compound has a very much higher affinity for CA I as compared to CA II, and this might be exploited for designing CA I-specific inhibitors based on such a zinc-binding function (see also Section 3.E).[14] By using compounds of type **108** as leads, several series of much stronger inhibitors were then reported, possessing modified sulfonamido moieties as zinc-binding functions, of the type SO_2NHOH,[108,109] SO_2NHCN,[110] $SO_2NHPO_3H_2$,[111] $SO_2NHSO_2NH_2$,[112] SO_2NHSO_3H[112], or $SO_2NHCH_2CONHOH$.[109]

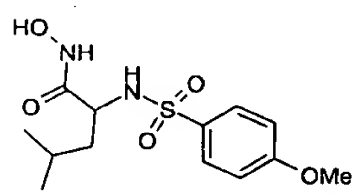






119

(K_i hCA I= 8 nM; hCA II = 11 nM; bCA IV = 13 nM)



120

(K_i hCA I= 50 nM; hCA II = 5 nM; bCA IV = 39 nM)

Thus, compounds such as **111–118**, possessing *N*-cyano, *N*-hydroxy, or *N*-phosphoryl-sulfonamido moieties, or the related modified sulfamide/sulfamic acid zinc-binding functions (in **116–118**), and diverse alkyl, aryl, or heterocyclic moieties in their molecules, showed affinities in the low nanomolar range for hCA II (except for **117**), being equipotent or better inhibitors than

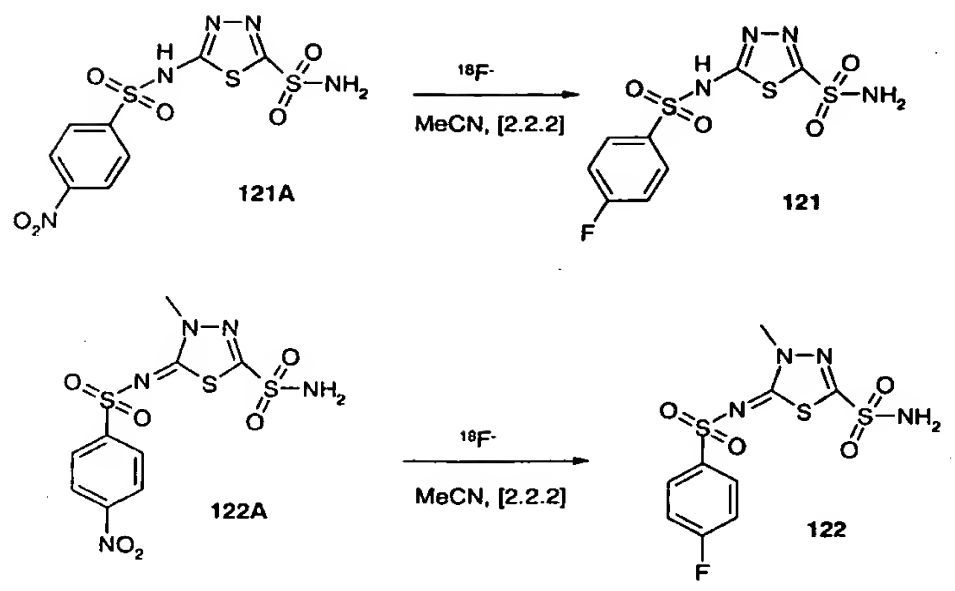
the corresponding unsubstituted sulfonamides.[108–112] Compound **117** is a weak inhibitor of hCA II (affinity constant of 1.2 μM), but it has a much higher affinity (50 nM) for hCA I, being thus one of the most "selective" hCA I inhibitors reported up to now (see also Section 3.E).[112]

Very recently, it was also shown that some sulfonylated amino acid hydroxamates also possess strong CA inhibitory properties.[109] Sulfonylated amino acid hydroxamates generally act as potent inhibitors of metalloproteases containing catalytic zinc ions, such as the matrix metalloproteinases (MMPs),[18,106,109,113,114] the bacterial collagenases[115,116] and other such enzymes. They bind to the Zn(II) ions present in these enzymes bidentately, coordinating through the hydroxamate (ionized) moiety.[113] Recently, Christianson's[117] group showed that two simple hydroxamates, of the type RCONHOH (R = Me, CF_3) act as micromolar inhibitors of hCA II, and bind to the Zn(II) ion of this enzyme, as demonstrated by X-ray crystallography. By using these two derivatives as lead molecules, we[109] designed a series of sulfonylated amino acid hydroxamate derivatives possessing the general formula $RSO_2NHCH(R')CONHOH$ and showed that they bind to the Zn(II) ion of CA, by means of electronic spectroscopic studies on the Co(II)-substituted CA. Some of these compounds, such as **119** and **120**, showed affinity in the low nanomolar range for the major CA isozymes (CA I, II, and IV), but substitution of the sulfonamide nitrogen by a benzyl or a substituted-benzyl moiety led to a drastic reduction of the CA inhibitory properties, and to an enhancement of the MMP inhibitory properties.[109] Thus, between the two types of zinc enzymes, the zinc proteases and the CAs, there exist some cross-reactivity from the point of view of the hydroxamate inhibitors, but generally strong MMP inhibitors are weak CAIs, and vice-versa.[109]

All these data demonstrate that in addition to the classical sulfonamide inhibitors, potent CAIs may be designed from other classes of compounds too, a fact that may be relevant for obtaining diverse pharmacological agents that modulate the activity of these wide-spread enzymes.

K. Diagnostic Tools for PET (Positron Emission Tomography)

Acetazolamide (**4**) is increasingly used as a diagnostic tool[2] since inhibition of brain CAs causes a selective increase of cerebral blood flow, with the concomitant rise in the carbon dioxide partial pressure,[118] and for this reason based on both magnetic resonance imaging (MRI) as well as positron emission tomographic (PET) have been devised to develop diagnostic tools that exploit these valuable properties of the drug.[118] The "acetazolamide-test" has become widely used in the assessment of cerebrovascular reactivity.[118,119] In most reported methods, resting- or acetazolamide-induced cerebral blood flow (CBF) is measured by PET procedures, in which positron emitting isotopes, such as ^{99m}Tc, ^{15}O, or ^{123}I were employed.[2,119] Although a small amount of radionuclide is involved in such measurements, they are easy to perform, accurate and extremely helpful in detecting regional abnormalities of hemodynamic reserve in cerebrovascular disease.[2,118,119] Since the number of aging people has increased very considerably (at least in the Western countries), additional studies have been performed for developing more specific CAIs for PET studies.[120,121] Many physiological and metabolic processes can also be studied with PET, provided that ligands, containing a suitable positron emitting nuclide, active toward specific receptors or enzymes are available.[2] Few CAIs labeled with positron emitting isotopes have been reported.[120,121] Thus, Le Bars et al.[120] worked up the radiosynthesis of ^{11}C-labeled acetazolamide, mainly used to study lung CA inhibition. Indeed, lung CA isozymes are known to play a critical role in the bicarbonate dehydration reaction with formation of the CO_2 eliminated in the expired air.[1,2] Nevertheless, to date the role of different CA isozymes in such processes escaped quantitative determination, although CA II and CA IV are the most abundant isozymes in this organ.[1,2] Since acetazolamide is an unselective CAI, our group[121] reported the synthesis of several benzolamide-like ^{18}F-labeled sulfonamide CAIs of the type **121** and **122**, which have the advantage over ^{11}C-acetazolamide in being more potent and more selective CAIs (over the membrane-bound *vs.* the cytosolic isozymes), and possessing a much longer half-life (^{11}C is a very short-lived positron emitting isotope, with $t_{1/2}$ = 20.4 min).[80,121]

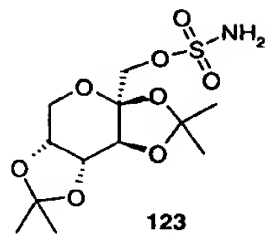


These inhibitors (**121**, **122**) have been prepared by aromatic nucleophilic substitution reactions between the nitro-derivatives **121A**, **122A** and radioactive fluoride, and act as nanomolar inhibitors of hCA II and bCA IV.[121] Studies with radiolabeled CAIs may help the understanding in greater detail of the physiological function of many CA isozymes, but little work in this field has been done up to now.

L. Antiepileptic Sulfonamides and Miscellaneous Other Inhibitors

Several sulfonamide CA inhibitors such as acetazolamide **4**, methazolamide **5**, topiramate **123**, or zonisamide **124** were and are still used as antiepileptic drugs.[122] The anticonvulsant effects of these or related sulfonamides are probably due to CO_2 retention secondary to inhibition of the red cell and brain enzymes, but other mechanisms of action, such as blockade of sodium channels and kainate/AMPA receptors, as well as enhancement of GABA-ergic transmission, were also hypothesized/proved for some of these drugs.[122] Acetazolamide and methazolamide are still clinically used nowadays in some forms of epilepsy, but they are considered to belong to a minor class of antiepileptic agents, whereas the recently developed drug, topiramate **123**, which is a very effective antiepileptic, was also shown to act as a strong CA inhibitor, with a potency similar to that of acetazolamide against the physiologically important isozyme CA II.[122]

Recently, a series of aromatic/heterocyclic sulfonamides incorporating valproyl moieties were prepared, in order to design antiepileptic compounds possessing in their structure two moieties known to induce such a pharmacological activity: valproic acid (**125**), one of the most widely used antiepileptic drugs, and the sulfonamide residue included in acetazolamide and topiramate, two CAIs with antiepileptic properties.[122] The valproyl derivative of acetazolamide (5-valproylamido-1,3,4-thiadiazole-2-sulfonamide; **126**) was one of the best hCA I and hCA II inhibitors in the above mentioned series, and exhibited very strong anticonvulsant properties in a MES test in mice.[122] In consequence, other 1,3,4-thiadiazole-sulfonamide derivatives possessing potent CA inhibitory properties, and substituted with different alkyl/arylcarboxamido/ sulfonamido/ureido moieties in the 5 position have been investigated for their anticonvulsant effects in the same animal model. It was observed that some structurally related derivatives (of types **127–135**), such as 5-benzoylamido-, 5-toluenesulfonylamido-, 5-adamantylcarboxamido-, and 5-pivaloylamido-1,3,4-thiadiazole-2-sulfonamide also showed promising *in vivo* anticonvulsant properties, and that these compounds may be considered as interesting leads for developing anticonvulsant or selective cerebrovasodilator drugs.[122]



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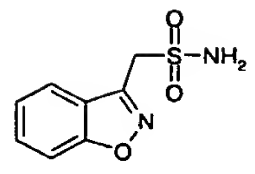

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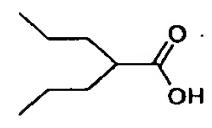

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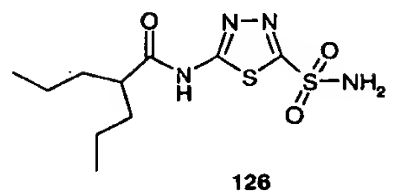

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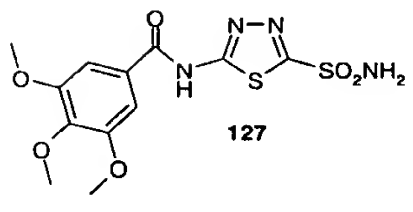

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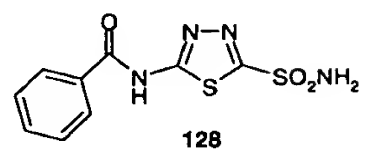

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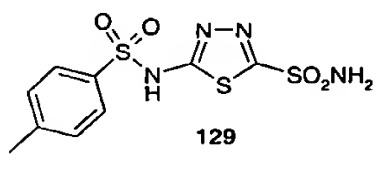

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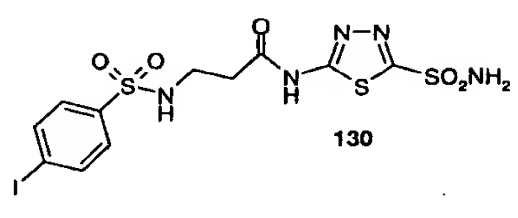

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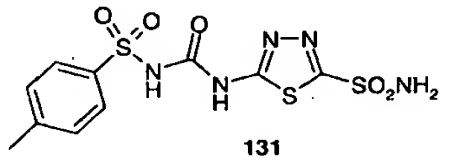

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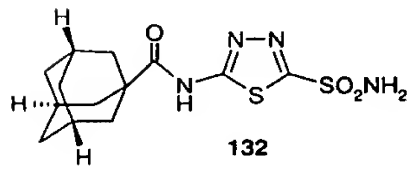

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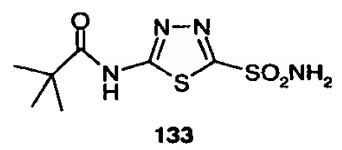

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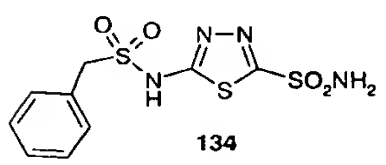
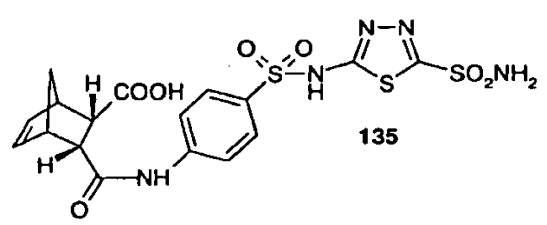
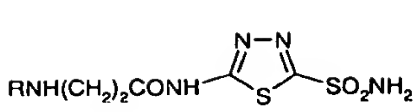


134

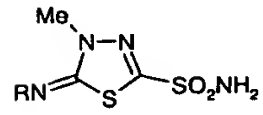


135

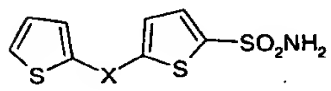
Other miscellaneous CAIs possessing interesting properties have also been reported. Thus, in an attempt to obtain gastric mucosa CA-specific CAIs, a group of sulfenamido-sulfonamides of type **136–139** have been reported[123] to possess powerful CA II and CA IV inhibitory properties. These compounds were designed in such a way as to liberate aromatic/heterocyclic sulfonamides and sulfenyl chlorides in the presence of gastric hydrochloric acid. The liberated compounds would then inhibit the enzymes involved in gastric acid production: the sulfonamides would bind within the CA active site, whereas the sulfenyl chlorides would inactivate (in an omeprazole-like manner) the gastric H^+/K^+-ATP-ase, by alkylating critical cysteine residues of this enzyme.[123]



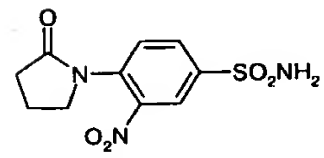
136: R = 2-O_2N-C_6H_4S
137: R = 4-O_2N-C_6H_4S



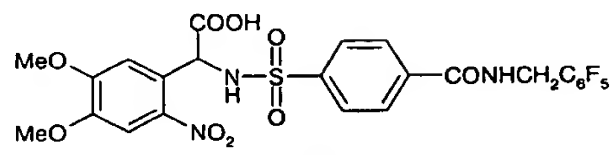
138A: R = 2-O_2N-C_6H_4S
139: R = 4-O_2N-C_6H_4S



140: X = S (K_I = 0.9 nM, hCA II)
141: X = SO_2 (K_I = 0.8 nM, hCA II)

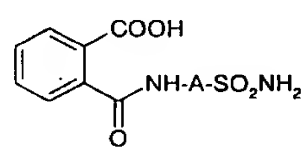


142: (K_I = 0.6 nM, hCA II)



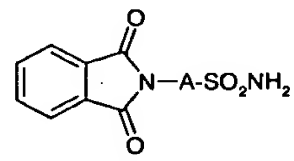
143

Klebe's group[124] detected several potent hCA II inhibitors by means of computer aided drug design. Three of these compounds, **140–142** showed nanomolar affinity to hCA II, and the X-ray structure has also been resolved for two such adducts.[124] Jain's group[125] on the other hand reported a caged hydrophobic CAI of type **143**, possessing a photolabile group derived from *o*-nitrophenylglycine. It is stated that such compounds might be useful for the site-specific delivery of prodrugs, for instance to the eye, but the high energy UV radiation needed to liberate the active inhibitor (p-H_2NO_2S-$C_6H_4CONHCH_2C_6F_5$) would probably do more harm than good to the eye tissues.[125]

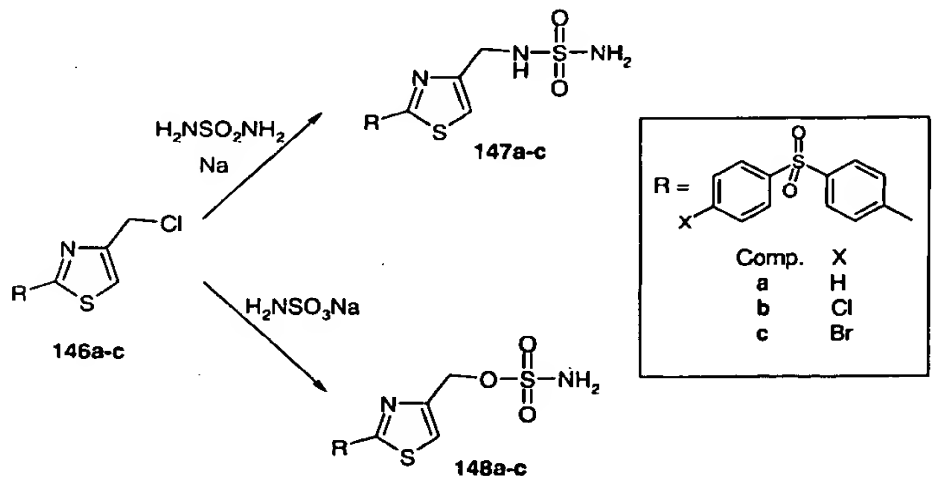


144

(A= aromatic/heterocyclic moiety, as in **A-T**)



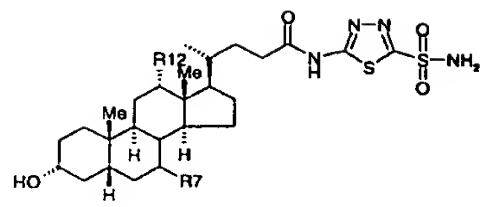
145



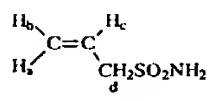
146a-c

147a-c

148a-c



149 (R7, R12 = H, OH, O-TBDMSi)



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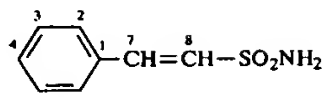
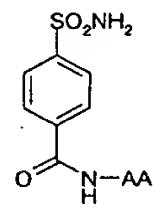
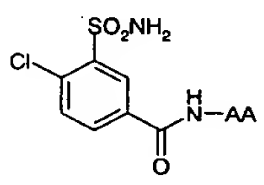


151



152



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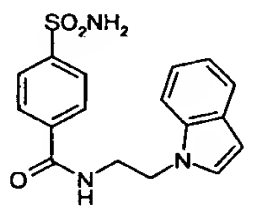


154

Sulfonamides incorporating 2-carboxy-benzenecarboxamido (phthaloyl) moieties in their molecules, of types **144**, were prepared by reaction of phthalic anhydride with aromatic/heterocyclic sulfonamides A–T under mild conditions, whereas a closely related series of derivatives was prepared by reaction of the same reagents under more energetic conditions, when the corresponding phthalimides **145** were obtained. Some of these compounds showed very good *in vitro* CA isozymes I, II, and IV inhibitory properties, with affinities for the enzymes in the low nanomolar range for the best inhibitors.[126]

A small series of 2-[4-(4-substituted-phenylsulfonyl)-phenyl]-4-chloromethyl-thiazoles **146** has been used as scaffold for the preparation of CAIs incorporating zinc binding functions of the sulfamide and sulfamate type.[127] The binding functions have been introduced in the molecules of these compounds, by reaction of the chloromethyl derivatives **146** with sodium sulfamide/sodium sulfamate, leading to the new CAIs **147** and **148**, respectively.[127] The new sulfamide/sulfamates were effective (nanomolar) CA II and CA IV inhibitors, but showed no inhibitory activity against isozyme CA I.[127]

Reaction of *tert*-butyl-dimethylsilyl (TBDMS)-protected bile acids (cholic, chenodeoxycholic, deoxycholic, lithocholic, ursodeoxycholic acids) or dehydrocholic acid with aromatic/heterocyclic sulfonamides possessing free amino/hydroxy moieties, of types A–T, in the presence of carbodiimides, afforded after deprotection of the OTBDMS ethers, a series of sulfonamides incorporating bile acid moieties in their molecules. Many such derivatives, among which **149** are illustrative representatives, showed strong inhibitory properties against three isozymes CA I, II, and IV.[128] Some of the most active derivatives, incorporating 1,3,4-thiadiazole-2-sulfonamide or benzothiazole-2-sulfonamide functionalities in their molecules, showed low nanomolar affinity for CA II and CAIV. Furthermore, the bioavailability of these derivatives in rabbits was shown to be comparable to that of acetazolamide, being in the range of 85–90%, showing them as promising candidates for systemically acting CA inhibitors.[128]

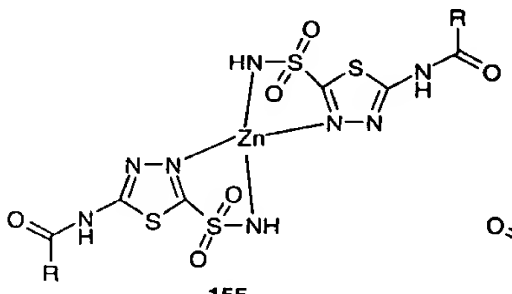
Unsaturated primary sulfonamides have only recently been investigated for their interaction with CA.[129] It was shown that such compounds, and more precisely allyl-sulfonamide **150** and *trans*-styrene sulfonamide **151** behave as nanomolar inhibitors of the physiologically relevant isozymes CA I and CA II.[129]

Reaction of 4-carboxy-benzenesulfonamide or 4-chloro-3-sulfamoyl benzoic acid with carboxy-protected amino acids/dipeptides, or aromatic/heterocyclic sulfonamides/mercaptans afforded two

series of strong sulfonamide CAIs, of types **152**, **153**.[130] Some of the new derivatives showed affinity in the low nanomolar range for isozymes CA II and IV, involved in aqueous humor secretion within the eye, and were tested as topically acting anti-glaucoma agents, in normotensive and glaucomatoous rabbits. Good *in vivo* activity and prolonged duration of action has been observed for some of these derivatives, as compared to the clinically used drugs dorzolamide and brinzolamide. Some of the reported 4-chloro-3-sulfamoyl benzenecarboxamides showed higher affinity for CA I than for the sulfonamide avid isozyme CA II.[130] The same type of amides were also reported thereafter by Grzybowski et al., with derivative **154** showing picomolar affinity to hCA II.[131]

M. Metal Complexes of Sulfonamides as CA Inhibitors

The presence of a multitude of heteroatoms (nitrogen and/or sulfur) in the molecules of heterocyclic sulfonamides such as acetazolamide (**4**), methazolamide (**5**), ethoxzolamide (**6**), dorzolamide (**8**), etc., make them attractive ligands for the complexation of metal ions.[26,132] Indeed, many metal complexes of these and structurally related heterocyclic sulfonamides have been reported,[132–148] characterized by spectroscopic and X-ray crystallographic methods,[134–148] and investigated for the inhibition of different isozymes,[133–148] as well as for potential applications as diagnostic tools/pharmacological agents.[143–148] Much investigated as ligands were the thiadiazole sulfonamides, such as acetazolamide, methazolamide and benzolamide. In fact, X-ray crystal structures are available only for metal complexes of these three sulfonamides.[132,135,141,142] Although ethoxzolamide and dorzolamide complexes have also been prepared,[132,134,137] their three dimensional structures have not been reported. Metal ions incorporated in such complexes mainly included transition metal ions such as Zn(II), Cu(II), Co(II), Ni(II), lanthanides(III), etc (for a review of the diverse metal ion derivatives of sulfonamides see Ref.[132]). The most interesting fact regarding these metal complexes of sulfonamide CAIs is that they act as 10–100 times more potent inhibitors of isozymes CA I and CA II as compared to the parent sulfonamide from which they were obtained, and this has been rationalized



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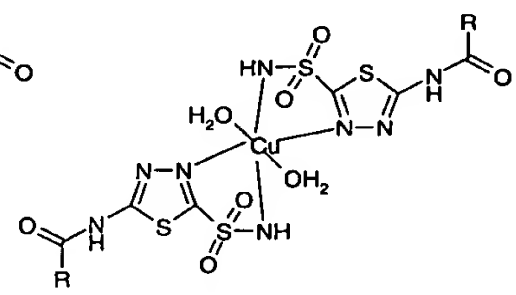

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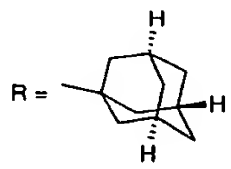
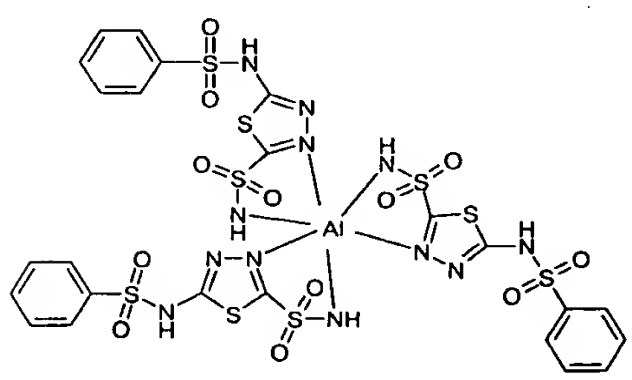




157

from the mechanistic point of view.[133] Thus, it is believed that this powerful inhibition is due to a dual mechanism of action, through sulfonamide anions, and metal ions, obtained in dilute solution by dissociation of the coordination compounds. Sulfonamide anions formed in this way then bind to the Zn(II) ion within the enzyme active site, whereas the metal ions block the proton shuttle residues of CA, i.e., His 64 for isozyme II, His 64 and 67 for isozyme I, and probably the entire histidine cluster in the case of isozyme II, as well.[10,26,133]

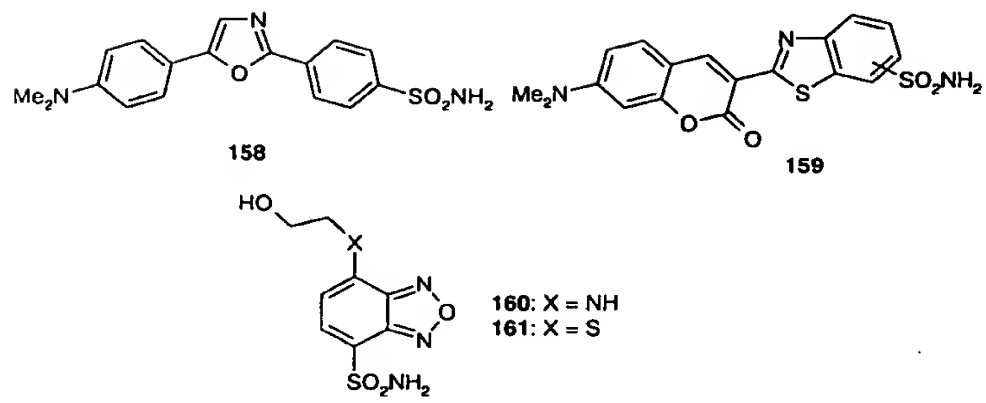
Several interesting applications have been reported for some metal complexes of heterocyclic sulfonamides possessing powerful CA inhibitory properties. Thus, zinc or copper complexes of a highly lipophilic thiadiazole sulfonamide, of types **155** and **156** (acting as nanomolar CA II and CA IV inhibitors) were very efficient IOP lowering agents when administered topically in normotensive or glaucomatous rabbits, although the parent sulfonamide from which the complexes were prepared does not possess topical antiglaucoma activity.[144] It was then shown that many other such metal complexes show topical activity,[144–146] and this has been explained by a modulation on the part of the metal ion of the physico-chemical properties of the complex, which in some cases becomes more polar and thus penetrates better through the cornea for inhibiting the ciliary processes CAs (see Section 3.D for details). It should also be mentioned that metal complexes are much more potent inhibitors as compared to the ligand sulfonamides, and this might also explain their strong topical activity as antiglaucoma agents.[143–146]

Some aluminum sulfonamide complexes, such as the benzolamide complex **157**, were shown to act as efficient antisecretory agents in dogs.[147] Gastric acid secretion parameters 3 days after treatment with such CAIs, (compound **157** or the corresponding Zn(II), or Mg(II) benzolamide/acetazolamide/ethoxzolamide complexes), in doses of 2 × 500 mg, twice a day, in dogs with chronic gastric fistulas, led to the observation that the gastric acid parameters BAO (the basal acid output), and MAO (the maximal acid output after stimulation with histamine) were drastically reduced, as compared to the same parameters in animals that did not receive these enzyme inhibitors.[147] It has been proposed that the Zn(II), Mg(II), and Al(III) sulfonamide complexes (eventually in combination with trace amounts of the corresponding copper derivatives) might constitute a new class of antiulcer agents, acting probably by a double mechanism: neutralization of hydrogen ions by a normal acid–base neutralization reaction (due to the presence of the metal ion derivative), coupled with a strong inhibition of carbonic anhydrase isozymes present in the gastric mucosa (due to both the sulfonamide and metal ion components of the drug), followed by reduction of formation of H^+ ions due to CO_2 hydration. Since the metal complexes mentioned above act as 10–100 times more effective CAIs as compared to the parent sulfonamides,[26,133] the use of such clinical agents could also lead to fewer side effects as compared to acetazolamide (or ethoxzolamide) in the treatment of gastric acidity dysfunctions.[148]

Recently, a series of Co(II), Ni(II), and Cu(II) complexes of a Schiff-base sulfanilamide derivative have been reported to possess nanomolar affinity for the physiologically relevant isozymes CA II and CA IV.[149]

N. CA Inhibitors-Based Biosensors

A fluorescent transduction method for the assay of nano-, pico-molar amounts of Zn(II) has been reported by Thompson's group.[150–153] The approach is based upon the energy transfer from a fluorescent label (such as the sulfonamide CAIs of type **158–161**[150–153]) when bound to the enzyme. Sulfonamides do not bind to the zinc free, apo-CA.[2,21] The presence of zinc ions leads to re-constitution of the native zinc enzyme, which avidly binds the fluorescent sulfonamide inhibitors. Binding of the sulfonamide to the Zn(II) ion within the enzyme active site has as a consequence important changes in the intensity and anisotropy of the fluorescence. This change can be quantitated, leading to the determination of the fraction of enzyme with bound inhibitor, and in consequence to the determination of picomolar amount of zinc ions present in solution.[150–153]



This technique allowed the development of a fluorescence microscopy method for studying organotypic cultures of rat hyppocampus among others[153] and it provices the basis for detailed physiological studies in which CAIs with special properties play important roles.

4. FUTURE PROSPECTS OF CA INHIBITION

CAIs are widely used therapeutic agents in the management or prevention of many diseases.[1,2] This is mainly due to the wide distribution of the 14 vertebrate CAs in many cells, tissues and organs, where they play crucial physiological functions. Still, the available pharmacological agents are far from being perfect. They possess many undesired side effects, mainly due to their lack of selectivity for the different CA isozymes. Thus, the development of isozyme-specific or at least organ-selective sulfonamide inhibitors would be highly beneficial both for obtaining novel types of drugs, devoid of major side effects, as well as for many physiological studies in which specific/selective inhibitors would be valuable tools for understanding the physiology of these enzymes. Still, prospects for achieving such a goal are not very optimistic at present, due to the high similarity between several isozymes in their interaction with sulfonamide inhibitors. The most similar of the isozymes tend to be only CA II and CA IV, which seem to be also the predominant and most wide-spread isozymes in many tissues in which specific inhibition is required to be achieved. Some progress in this field has been recorded recently, as shown in Section 3.E, by the development of high molecular weight membrane-impermeant inhibitors, which being excluded from the intracellular space inhibit selectively only membrane-associated and not the cytosolic CA isozymes.

Important advancements have been noted in the past few years in the development of topically effective CAIs for the treatment of glaucoma, with two available drugs, dorzolamide (TRUSOPT™, Merck & Co.) and brinzolamide (AZOPT™, Alcon Laboratories), respectively. These drugs resolved a lot of the undesired side effects previously observed with the systemically used CAIs for the treatment of glaucoma. Both dorzolamide and brinzolamide are effective antiglaucoma agents, but they tend to pose tolerability problems in many patients, because of local side effects. This is probably due to the fact that these two compounds are salts of weak bases with a very strong acid, and thus the pH of the administered drug is relatively acidic. Thus, the search of novel types of topically acting antiglaucoma sulfonamides continues. Recently, a different (and more general) approach from that of Merck and Alcon has been reported for the preparation of topical antiglaucoma CAIs, which consists in introducing water-solubilizing tails to the molecules of aromatic/heterocyclic sulfonamides (Section 3.1.2). Several of the compounds obtained in this way, which can form salts with pH values both in the slightly acidic as well as slightly basic region (pH 6.5–7.5), showed very potent IOP lowering properties in an animal model of glaucoma, and some of them are in clinical evaluation. Furthermore, such agents showed a longer IOP lowering effect as compared to the first generation topically acting sulfonamides, which may constitute a valuable feature for a new drug from this family of pharmacological agents. Another important aspect in the reviewers view for the future applications

of CAIs in ophthalmology regards their possible use in the treatment of macular edema and related degenerative pathologies, for which no effective treatment is known at the moment.[1,2] Several preliminary data seem to indicate the value of topically and systemically acting sulfonamides in improving visual function in patients with macular degenerative disease.[1,2]

The potential of CAIs in the treatment of epilepsy and other neurological/neuromuscular disorders is far from being fully exploited. Thus, no specific brain enzyme CAI has been reported up to now, and a specific cerebrovasodilator from this class of pharmacological agents would be a valuable drug and also an interesting diagnostic tool, since acetazolamide is widely employed (the so-called "acetazolamide-test") for the assessment of cerebrovascular reactivity in normal and pathological states (Section 3.K). Even so, the "imperfect" drug which acetazolamide is, represents anyhow the only available therapy of choice in many minor neurological disorders (such as familial hemiplegic migraine and ataxia; tardive dyskinesia; hypo- and hyperkalemic periodic paralysis, etc.[1,2]). The development of more selective CAIs of this type would be an interesting challenge for medicinal chemists and pharmacologists.

A gastric selective CAI could also be helpful in the treatment of gastric/duodenal dysfunctions correlated with gastric acid secretion imbalances. Although many pharmacological agents are presently used in the treatment of such conditions, all of them possess, in different degrees, undesired side effects, and thus, a place for a sulfonamide CAI would be possible to find in the armamentarium of antiulcer therapeutic agents.

Sulfonamide CAIs played a crucial role in the understanding of renal physiology and pharmacology, and led to the development of widely used diuretic drugs such as the benzothiadiazine and high ceiling diuretics. Still, few advances have been needed to date in this field, perhaps also because a large number of clinically approved diuretics are already available. With all this, an orphan drug of this class still waits a wider (and really deserved) use, benzolamide. This compound, although relatively similar to acetazolamide, possesses a different pharmacology, and might be successfully employed as a selective renal CAI.[1,2] Its use in chronic obstructive lung disease or in acute mountain sickness seems to be superior to acetazolamide, and more detailed studies in these areas are needed. Few studies are also available on the therapeutic potential of CAIs in osteoporosis.[1,2] The development of bone-targeted sulfonamides would lead to drugs devoid of severe systemic effects, but little progress has been noted in this field.

A recent and new field in CAI research has been opened by the report of the potent anti-tumor properties of an entire class of sulfonamide CAIs, as well as by the isolation of some CA isozymes predominantly present in tumor cells (Section 3.I). Although the mechanisms by which sulfonamides inhibit tumor cell growth are not sufficiently understood at the moment, we predict important advancements in this direction, since several laboratories (our own included) are involved in the synthesis, evaluation, and *in vitro/in vivo* antitumor testing of novel classes of sulfonamides with potential application as anticancer therapeutic agents.

The development of diagnostic tools based on CAIs is also an attractive future research direction, both for the development of MRI as well as PET agents. Some recent reports showed that radiolabeled potent CAIs may be easily synthesized and they should be evaluated for *in vivo* applications soon. They should be particularly useful for lung imaging studies, since inexplainably, this is one of the organs most difficult to "visualize" by means of PET techniques. The development of a positron emitting isotope labeled CAI that is membrane-impermeant would be very helpful for such a purpose.

No pharmacological agents from this class of compounds has so far been developed for inhibiting the liver enzyme (predominantly CA V), and since this is involved in biosynthetic reactions (ureagenesis; glucogenesis, etc) such a therapy might be useful in some metabolic disfunctions. In fact, CA V remains one of the least studied "major" CA isozymes, and even its catalytic mechanism is not fully understood. For instance, the rate-limiting proton transfer step of the catalytic cycle, which for CA II is assisted by the active site residue His 64, cannot be performed by Tyr 64, the amino acid present in CA V. We propose that this rate-determining catalytic step is assisted by the many arginine

residues present on the rim and surface of the active site cavity. These highly basic residues may shuttle protons easily due to the presence of the guanidino moiety (it has also been proved that arginine is a powerful activator of CA I and CA II).[97]

Very few studies are available regarding the inhibition of non-vertebrate CAs. Since CAs were recently shown to be present in a multitude of eubacteria and *Archaea*, it should be possible to develop CAI-based antibiotics, a field that raised much interest some years ago, with promising results in the use of ethoxzolamide for the treatment of meningitis.[154–157] This type of inhibition has also been exploited for developing selective culture medium for other pathogenic bacteria, such as *Branhamella catarrhalis*,[156] in the presence of different *Neisseria species*. Some strains of *Pseudomonas*, *Staphylococcus*, *Streptococcus*, *Serratia*, and *Proteus*, were reported[156] to strongly express a gene product immunologically related to CA. On the other hand, α-, β-, and γ-CAs have been purified in at least six species of Bacteria: *Neisseria spp.*, *E. coli*, *Synechocystis spp.*, *Acetobacterium woodi*, *Anabaena variabilis*, and *Rhodospirillum rubrum*, but it is established that these enzymes are nearly ubiquitous in prokariotes.[157] It is thus envisageable that sulfonamide CAIs able to inhibit the growth of such bacteria (some of which are feared pathogens, which developed resistance to classical antibiotics)[158] might be developed, leading to novel types of pharmacological agents useful in the fight against infections. Finally, very little is known regarding inhibition of CAs present in Archaea (γ-CAs have been isolated in *Methanosarcina thermophila* and *Methanobacterium thermoautotrophicum* at the present time),[157] but it may be speculated that potent and selective inhibitors for such enzymes might be developed, leading to interesting novel biomedical applications.[1,2,158] Furthermore, the recent report of parasitic CAs by Krungkrai et al.[159] who discovered the presence of at least two different CAs in *Plasmodium falciparum*, the malaria provoking protozoa, opens new vistas to the development of pharmacological agents based on such enzyme inhibitors. Red cells infected by *Plasmodium falciparum* contained CA contents approximately twofold higher than those of normal red cells.[159] The three developmental forms of the asexual stages (i.e., ring, trophozoite, and schizont) were isolated from their host red cells and found to have stage-dependent CA activity. The enzyme was then purified to homogeneity, showing a M_r of 32 kDa, being active in monomeric form (the human red cell enzyme was also purified for comparison with the parasite enzyme in this study).[159] The parasite enzyme activity was sensitive to well-known sulfonamide—CAIs such as sulfanilamide and acetazolamide. The kinetic properties and the amino terminal sequences of the purified enzymes from the parasite and host red cell were found to be different, indicating that the purified protein was a distinct protein, i.e., *P. falciparum* CA. In addition, the above mentioned enzyme inhibitors showed an antimalarial effect against *in vitro* growth of *P. falciparum*. This very important contribution[159] shows that CAIs may represent valuable future drugs for the treatment of malaria.

The conclusion is that these enzymes and their inhibitors are indeed remarkable; after many years of intense research in this field, they continue to offer interesting opportunities for the development of novel drugs, new diagnostic tools, or for understanding in greater depth of the fundamental processes of the life sciences.

ACKNOWLEDGMENTS

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LABORATORY INVESTIGATION

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Carbonic anhydrase inhibition increases retinal oxygen tension and dilates retinal vessels

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Abstract *Background:* Carbonic anhydrase inhibitors (CAIs) increase blood flow in the brain and probably also in the optic nerve and retina. Additionally they elevate the oxygen tension in the optic nerve in the pig. We propose that they also raise the oxygen tension in the retina. We studied the oxygen tension in the pig retina and optic nerve before and after dorzolamide injection. Also the retinal vessel diameters during carbonic anhydrase inhibition were studied. *Methods:* A polarographic oxygen electrode was placed transvitreally immediately over the retina or the optic disc in anaesthetised pigs. The oxygen tension was recorded continually and 500 mg dorzolamide was injected intravenously. Retinal vessel diameters were analysed from monochromatic fundus photographs taken before and after injection of dorzolamide. *Results:* Baseline retinal oxygen tension (RPO_2) was 3.34±0.50 kPa (mean ± SD, n=6) and baseline optic nerve oxygen tension ($ONPO_2$) was 3.63±1.00 kPa. RPO_2 was increased by 0.36±0.11 kPa (n=6, P=0.025) and $ONPO_2$ by 0.73±0.34 kPa (n=6, P=0.003) 30 min after dorzolamide administration. The retinal arterioles was significantly dilated by 13±7% (n=5, P=0.016) and the retinal venules by 12±8% (n=5, P=0.030) 30 min after injection of dorzolamide. *Conclusion:* Retinal and optic nerve oxygen tension increased with systemic administration of dorzolamide. The retinal vessels dilated, probably causing increased blood flow inducing the observed increase in RPO_2. The increased oxygenation of retina by CAI may offer therapeutic possibilities in ischaemic diseases of the retina and optic nerve.

Introduction

Carbonic anhydrase inhibitors (CAIs), when administered systemically, increase blood flow in the central nervous system [4, 6, 24] and also increase the oxygen tension (PO_2) in the brain [3, 11]. It is widely debated whether CAIs, used either topically or systemically, dilate retinal vessels [8, 14] and increase retinal blood flow [7, 10, 17]. We have previously shown that CAIs such as acetazolamide and dorzolamide elevate the optic nerve PO_2 ($ONPO_2$) [20] in pigs and proposed that this may be due to increased blood flow and vasodilatation, and that this effect could have implications in the pharmacological treatment of glaucoma.

It has also been suggested that pharmacological elevation of retinal PO_2 (RPO_2) could be important in the treatment of ischaemic diseases of the retina, such as retinal vein occlusions [12, 15, 19] and retinopathy of

prematurity [22]. It is possible that CAIs have a role in the treatment of these diseases. Therefore, we studied the effect of systemically administered dorzolamide on the RPO_2 in the effort to ascertain whether dorzolamide might represent a potential pharmacological treatment for retinal hypoxia and ischaemia.

By using a known and reproducible method for analysing retinal vessel diameters [2, 5] we additionally investigated whether the diameters of the retinal vessels changed when CAI was administered. Thus, changes in RPO_2 and retinal vessel diameters were compared to see whether these two factors were directly correlated.

Materials and methods

Domestic pigs (Danish Landrace, n=17, 28–30 kg body weight), brought up in a specific pathogen-free environment were used as experimental animals. Their treatment was supervised by a veterinarian nurse and the "Principles of laboratory animal care" (NIH publication No. 85–23, revised 1985), the OPRR Public Health Service Policy on the Humane Care and Use of Laboratory Animals (revised 1986) and the U.S. Animal Welfare Act, were followed. Permission for the use of pigs in this study was granted by Dyreforsøgstilsynet (the Danish animal experiments inspectorate).

Dorzolamide hydrochloride was obtained from Merck, Sharp & Dohme, Glostrup, Denmark and was dissolved as a 3% solution in 100 mM citrate buffer, pH 5.6.

Anaesthesia and animal preparation

Induction of anaesthesia was obtained by i.m. injection of 15 mg midazolam (Dumex-Alpharma) followed by 3 ml of a mixture of tiletamine 25 mg/ml and zolazapam 25 mg/ml (Zoletil 50 vet, Boehringer Ingelheim), xylazine 13 mg/ml (Narcoxyl vet, Veterinaria), ketalar 15 mg/ml (Ketaminol, Veterinaria) and methadone 2.5/ml (Methadon DAK, Nycomed). After induction, pigs were intubated and artificially ventilated with air. Catheters were placed in the left femoral artery, the left femoral vein and the left superficial epigastric vein. During the experiment, anaesthesia was maintained by infusion of pancuronium bromide 8 mg/h (Pavulon, Organon) and fentanyl 400 μg/h (Fentanyl, Dumex-Alpharma) in one vein and pentobarbital 300 mg/h (Mebumal, Den Kongelige Veterinaer- og Landbohøjskoles Apotek, Denmark) in the other vein. A pressure transducer was connected to the arterial catheter for continuous measurements of arterial blood pressure (MAP). Heart rate (HR) was recorded from electrodes placed on the animal. The signals from arterial pressure transducer, rectal temperature probe and ECG were sampled continuously by an A/D converter (Digidata 1200B, Axon Instruments, Union City, CA, USA) and digitally registered with the Axoscope 8.1 program (Axon Instruments). The sampling rate was 10 kHz.

The pig was placed in a sling, the head was secured stereotactically and a speculum was placed between the eyelids of the left eye.

The pupil of the left eye was dilated with 1% atropine (Atropin SAD, Sygehusapotekerne, Denmark), 0.4% oxybuprocaine (Oxybuprokain SAD, Sygehusapotekerne), 1% cyclopentolate (Cyclogyl, Alcon) and 2.5% methaoxedrine (methaoxedrine SAD, Sygehusapotekerne) eye drops. Two 4-0 silk traction sutures were placed in the sclera to immobilise the eye. A sclerotomy was made 2.0 mm behind the corneal limbus in the superior nasal quadrant and a plastic cannula (16 gauge) was placed in the sclerotomy.

Oxygen tension experiments

The PO_2 over either the retina (n=6) or the optic nerve (n=6) was measured with a polarographic oxygen electrode mounted with an internal Ag/AgCl reference electrode inside a 20-gauge needle (model 818, Diamond General Development, Ann Arbor, MI, USA) [13, 20]. To avoid contamination of the electrode with blood products, it was advanced through the cannula placed in the sclerotomy. The signal from the electrode was measured continuously with a chemical micro sensor (model 1231, Diamond General Development). Aided by a micromanipulator and visualised by indirect ophthalmoscopy, the tip of the electrode was placed 0.5 mm over the retina/optic disc either 3 mm superior to the optic nerve head, where no vessels were seen, or over an avascular area of the optic nerve. The diameter of the electrode was 20 μm. The oxygen electrode was calibrated before and after each experiment in 100% N_2 and 5% O_2/95% N_2 in a calibration cell (model 1251, Diamond General Development). The signal from the oxygen electrode was sampled by the A/D converter and digitally registered. Initially, arterial blood samples were drawn from the catheter in the femoral artery to evaluate the blood gas values; the blood samples were analysed for oxygen, carbon dioxide, and pH with a blood gas analyser (ABL 605, Radiometer, Copenhagen, Denmark). The respirator was adjusted in stroke volume and frequency to ensure normal values for arterial blood pH (apH) (7.38–7.42), PO_2 (aPO_2) (10–14 kPa) and PCO_2 ($aPCO_2$) (5.5–7.5 kPa). Subsequently a stable oxygen recording in the vitreous was obtained. The pig was given 100% oxygen in the inspiratory air to test whether the oxygen electrode reacted properly. During the rest of the experiment, the pig was ventilated with atmospheric air. After a stable PO_2 recording had been obtained in the vitreous, the pig was given 500 mg dorzolamide intravenously, and blood samples were taken at time points −10, −1, 1, 5, 10, 15, 20 and 30 min in relation to the injection of the drug.

The experiments were all run with room lights on (white fluorescent light tubes). The ambient light intensity at the level of the pig's eye was found to be approximately 500 lux.

The mean total duration of the experiments was 4 h and 36 min ±56 min SD (n=12). The electrode recordings lasted 3 h and 47 min ±72 min. The oxygen electrode drift was less than −0.1 kPa per hour.

Retinal vessel diameter measurements

Fundus photos of the pigs (n=5) were taken on Colour Diapositive Film (Elitechrome 200 ED-3, Kodak). Scanning of the photos was performed at 2700 dpi (Nikon Coolpix LS2000). Fundus photos that were not well focused were excluded from the analysis. The well-focused photos for every pig [on average three photos at each of the following times: before injection of dorzolamide (baseline), 0–10 min, 10–20 min and 20–30 min after injection] were aligned. The arterioles (120–180 μm in diameter) and venules (180–240 μm in diameter) were analysed and their diameter was determined (on average two arterioles and two venules per pig), using a specially developed macro program (NIH Image vers. 1.61, http://rsb.info.nih.gov/nih-image), measuring the diameter in pixels. Regions of interest were selected across straight vessels for a distance of about three vessel diameters approximately half a disc diameter from the optic disc (Fig. 1).

Cross-sectional profiles in the green band were extracted (Fig. 2). The borders of the vessels were located at 50% change from the surrounding background to the minimum value inside the vessel on each side of the bright centre reflex [2, 5].

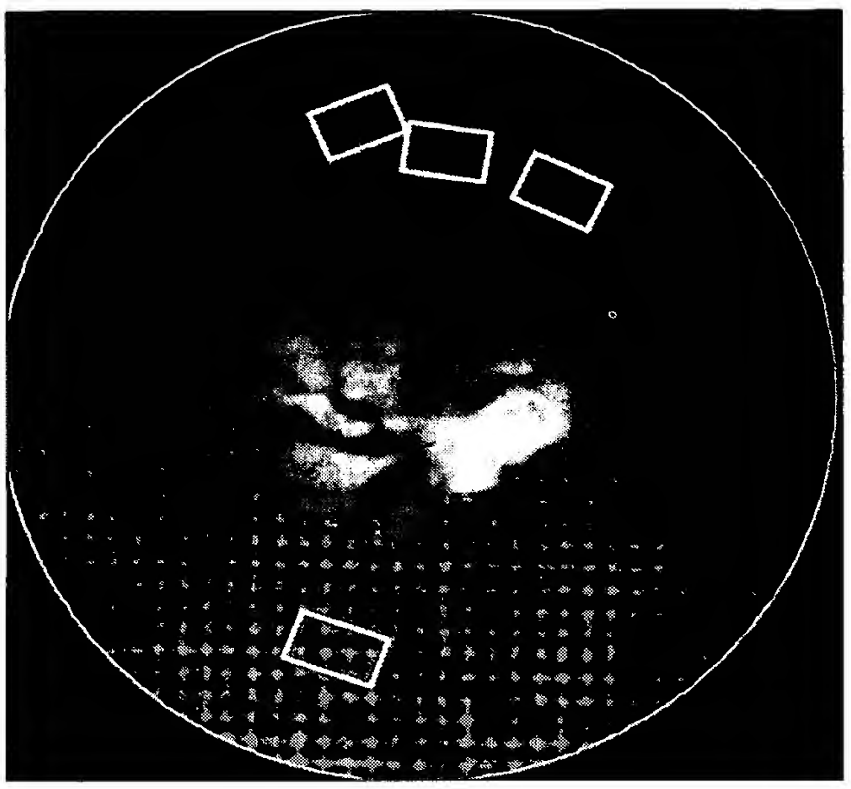

Fig. 1 Fundus photo of the pig eye before injection of dorzolamide. The *boxes* indicate where the vessel diameters were measured

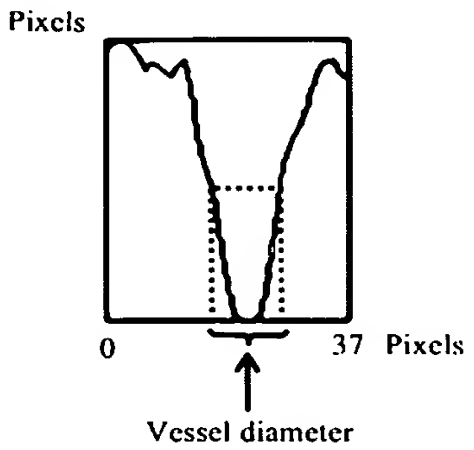

Fig. 2 A profile of a retinal vessel and a demonstration of how the retinal vessel diameter is measured

Data processing

RPO_2, $ONPO_2$, MAP and HR values (averaged over 15 s in the Axoscope recording) for baseline and time points after injection of the study drug were used for all the calculations. The mean and standard deviation of $ONPO_2$, RPO_2, apH, $aPCO_2$, aPO_2, MAP and HR were calculated (Table 1 and Fig. 3).

The vessel diameters for arterioles and venules were pooled in the four time intervals: before injection of dorzolamide (baseline) and 0–10 min, 10–20 min and 20–30 min after injection. The baseline diameters were given in pixels, while the diameters of the venules and arterioles at the different times were expressed in percent of baseline, showing the relative changes in the vessel diameters (Table 2). The precision of the observations was evaluated by the coefficient of variation (100%*SD/mean) of the baseline measurements of the vessels. This was multiplied with the mean pixel value of the baseline values to get the standard deviation in pixels.

Differences in the parameters before and after drug injections in each pig were tested for significance using Student's paired two-tailed *t*-test. Difference between groups was tested for significance using Student's unpaired two-tailed *t*-test. A significance level of 0.05 was used.

All results (Tables 1, 2) were presented as mean ± SD [*n*=number of experiments, *P*=probability of statistical significance (significance level <0.05)].

Graphic presentations of the tracings and the results were made in SigmaPlot 2001 for Windows (Version 6.00 SPSS, Chicago, USA).

Results

Oxygen studies

The baseline RPO_2 in the pig was 3.34±0.50 kPa (n=6). This significantly increased by 0.36±0.11 kPa (n=6, P=0.025) after an intravenous injection of 500 mg dorzolamide (Table 1 and Figs. 3, 4). The baseline $ONPO_2$ in the pig was 3.63±1.00 kPa. This significantly increased by 0.73±0.34 kPa (n=6, P=0.003) following intravenous

Table 1 Baseline and delta values (mean ± SD) 30 min after injection of 500 mg dorzolamide for parameters for the retinal and optic nerve experiments. The control values and changes (Δ) 30 min after administration of 500 mg dorzolamide of the oxygen tension in the eye (PO_2), either over the retina or over the optic nerve, arterial blood pH (apH), arterial blood PCO_2 ($aPCO_2$), arterial blood PO_2 (aPO_2), mean arterial pressure (MAP) and heart rate (HR)

	Retinal experiments (n=6)	Optic nerve experiments (n=6)	Unpaired t-test[a]
PO_2 (eye) baseline (kPa)	3.34±0.50	3.63±1.00	n.s.
ΔPO_2 (eye) (30 min) (kPa)	0.36±0.11*	0.73±0.34*	n.s.
apH baseline	7.41±0.01	7.41±0.01	n.s.
ΔapH (30 min)	−0.08±0.01*	−0.08±0.02*	n.s.
$aPCO_2$ baseline (kPa)	6.6±0.6	7.0±0.5	n.s.
Δ$aPCO_2$ (30 min) (kPa)	1.2±0.1*	1.4±0.6*	n.s.
aPO_2 baseline (kPa)	13.8±2.6	11.2±1.0	n.s.
ΔaPO_2 (30 min) (kPa)	−0.1±0.4	0.0±0.9	n.s.
MAP baseline (mmHg)	97±9	99±18	n.s.
ΔMAP (30 min) (mmHg)	−15±7*	−4±5	P=0.02
HR baseline	84±9	83±14	n.s.
ΔHR (30 min)	3±11	7±16	n.s.

* Changes over time significant (P<0.05, paired t-test) relative to baseline
[a] Comparison of retinal and optic nerve measurements: *n.s.* not significant ($P \geq 0.05$)

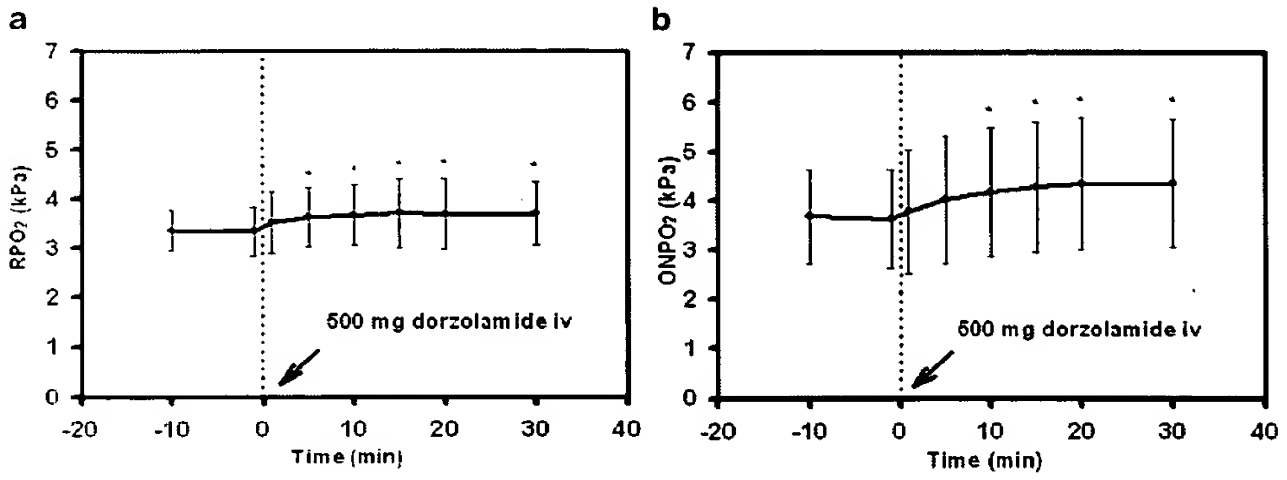

Fig. 3 a Oxygen tension over the retina (RPO_2) in kPa in the pig. Mean values and SD of RPO_2 at baseline and after intravenous administration of 500 mg dorzolamide (n=6) are shown. The injection of dorzolamide is at time = 0. There is a significant increase in RPO_2. *Changes over time significantly different (P<0.05, paired t-test) from baseline. **b** Oxygen tension over the optic nerve ($ONPO_2$) in kPa in the pig. Mean values and SD of $ONPO_2$ of baseline and after intravenous administration of 500 mg dorzolamide (n=6) are shown. The injection of dorzolamide is at time = 0. There is a significant increase in $ONPO_2$. *Changes over time significantly different (P<0.05, paired t-test) from baseline

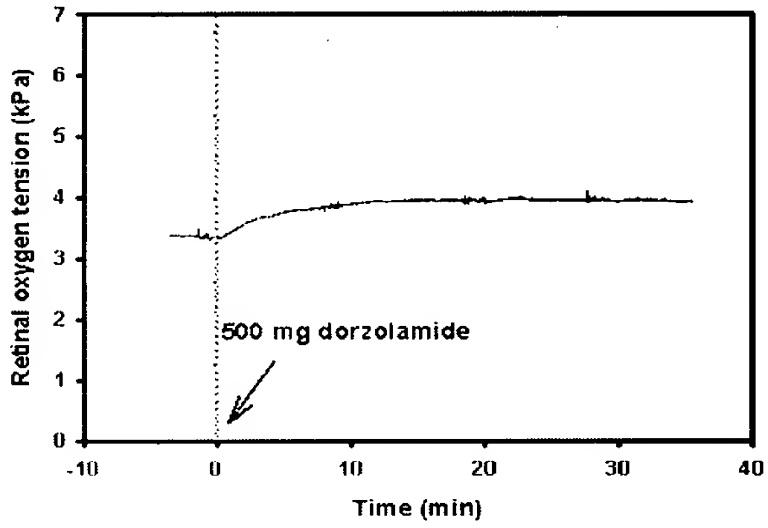

Fig. 4 Tracing of a recording of the oxygen tension over the retina (RPO_2) in kPa in the pig during a single representative experiment. The recording shows baseline and the time after intravenous administration of 500 mg dorzolamide. The injection of dorzolamide is at time = 0 min

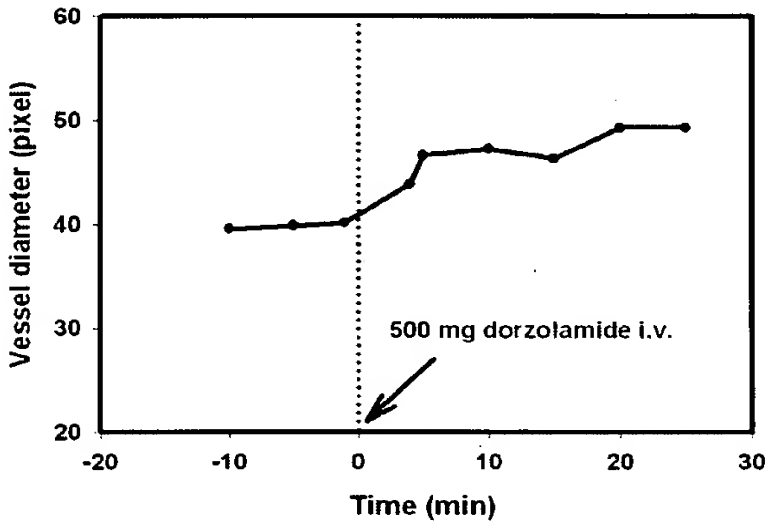

Fig. 5 The diameter of a retinal venule, given in pixels, during a single representative experiment where 500 mg dorzolamide was injected. The injection of dorzolamide is at time = 0 min

Table 2 Baseline value of retinal arteriolar and venular diameters and relative changes in diameters (in percentages of baseline diameter) of retinal arterioles and venules after injection of 500 mg dorzolamide in the periods 0–10 min, 10–20 min and 20–30 min after administration

	Arterioles	Venules	Unpaired t-test[a]
Baseline (pixels)	34±7	42±15	P=0.018
0–10 min (%)	4.3±1.0*	5.0±5.3	n.s.
10–20 min (%)	8.3±6.6*	6.4±2.9*	n.s.
20–30 min (%)	12.6±7.1*	12.2±8.2*	n.s.

* Changes over time significant relative to baseline (P<0.05)
[a] Comparison of the values in the two groups of vessels: *n.s.* not significant ($P \geq 0.05$)

injection of dorzolamide (Table 1 and Fig. 3). There was no significant difference in the baseline PO_2 between the retina and the optic nerve (Table 1). The increases in PO_2 after the injection of 500 mg dorzolamide also did not differ significantly between retina and optic nerve (Table 1). Additionally, the time courses were similar (Figs. 3, 4).

Vessel diameter studies

At 30 min after the intravenous injection of 500 mg dorzolamide, the retinal arterioles had dilated significantly by 13±7% (n=5, P=0.016) and the retinal venules by 12±8% (n=5, P=0.030) (Table 2). The course of a

single experiment (a retinal venule) is shown in Fig. 5. There was no significant difference in relative diameter changes between the arterioles and the venules.

The mean coefficient of variation for the baseline values was 2.9% (n=53), corresponding to an average of 1.1 pixels.

Discussion

Our data showed that both RPO_2 and retinal vessel diameter increased after intravenous injection of 500 mg dorzolamide. This is, to our knowledge, the first time that a direct relationship between vessel dilatation and an PO_2increase in tissue has been demonstrated. Additionally, the increase in RPO_2 suggests the possibility of pharmacologically improving the oxygenation of the retina.

The observed CAI-induced increase in RPO_2 [20] may be due to either decreased metabolism or increased oxygen supply of the retinal tissue, or possibly a combination of these two factors. It has been shown that 1 g acetazolamide given intravenously does not decrease the cerebral metabolic rate for oxygen in healthy humans [24]. Our results confirm this study by indicating that the increase in RPO_2 is caused by an increased oxygen supply through a significant vasodilatation of retinal vessels, probably causing increased blood flow.

We compared a non-invasive method of measuring retinal vessel diameters with invasive oxygen measurements. The standard deviation of the retinal vessel analyses was small compared to the changes we found in the retinal vessel diameters after CAI, indicating that our results were reliable. Non-invasively we measured a dilatation of the retinal vessels while we invasively measured an increase in the RPO_2, in both cases due to systemic carbonic anhydrase inhibition. This verifies that the PO_2 changes we measure with the invasive method probably also occur in the intact eye.

It has been shown that retinal vein diameter increases significantly by 1.9% during 1 h after the injection of 500 mg acetazolamide in healthy individuals [18]. This is less than we found in the pigs given 500 mg dorzolamide. The difference may be due to the fact that dorzolamide is more potent than acetazolamide in inhibiting the relevant carbonic anhydrase isozymes in the eye [20, 21]. Additionally, carbonic anhydrase may not have been fully inhibited in the human experiments because of the higher bodyweight of the humans compared to the pigs.

Carbonic anhydrase inhibition induces CO_2 accumulation in blood and tissue [23]. CO_2 is a known vasodilatory stimulus in cerebral vessels [9] and it was previously shown that CO_2 breathing induces increases in $aPCO_2$ and $ONPO_2$ similar to those induced by carbonic anhydrase inhibition [20]. The CAI-induced vessel dilatation and $ONPO_2/RPO_2$ increase is possibly due to this CO_2 accumulation. However, it has also been shown that acetazolamide directly dilates isolated resistance arteries [16]; thus, a direct vasodilatory effect cannot be excluded.

We have previously shown that measurements of the PO_2 in the normal pig vitreous can be obtained using the 20 μm oxygen electrodes employed in the present study [13, 20]. The vitreous acts as a diffusion medium with very low metabolism and oxygen gradient (about 2 mmHg per millimetre in vitreous) [1]. The measurements in front of the optic nerve and retina are therefore relatively insensitive to the exact location of the sensor. Also, oxygen molecules diffuse freely from the retinal vessels to the retina and to the vitreous. Therefore, we interpret the oxygen measurements in the vitreous as reflecting the average oxygen concentration in the vitreal part of the retina and optic nerve head, denoted retinal oxygen tension and optic nerve oxygen tension.

The dose of 500 mg dorzolamide in these experiments was used because earlier findings had shown that this dose gives near-maximal response in the $ONPO_2$, indicating a full inhibition and saturation of the carbonic anhydrase enzymes. This application is, of course, different from the typical topical application of these glaucoma drugs in humans. The aim was to discover the principal pharmacological effects of this drug on the pig optic nerve and later to proceed to studies with smaller clinical doses in the human glaucoma patient.

Table 1 shows that at normal values of apH, the arterial PCO_2 is higher than in humans. Additionally, pigs have a high standard base excess (between 2 and 6 mmol/l). To investigate whether this is normal in the pig we placed an arterial catheter in the carotid artery of three pigs, anaesthetized them and let them survive for 13 days in their habitual surroundings. We took blood samples twice a day and found that the blood gasses in unanaesthetised pigs were identical to the blood gasses in the anaesthetised pigs. Based on these results we concluded that high arterial PCO_2 and standard base excess must be normal for pigs.

The pigs used in this study have a severe astigmatism and individual refractive errors. Therefore, we used each vessel as its own control, expressing the diameter changes in percent of baseline. We know the approximate diameters of the vessels we investigated since in two pigs we introduced a cannula of a known size, placed it on the retina close to the vessels, and from photographs calculated the diameters of the average venules and arterioles in micrometres.

Carbonic anhydrase inhibition dilated retinal blood vessels and increased RPO_2 under normal conditions in pigs. It remains to be shown whether CAIs, administered systemically or topically, are capable of countering hypoxia and ischaemia in retinal and optic nerve diseases through dilation of retinal vessels. This effect might be beneficial in the treatment of these diseases.

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Optic Nerve Oxygen Tension in Pigs and the Effect of Carbonic Anhydrase Inhibitors

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Progression of Retinopathy with Intensive versus Conventional Treatment in the Diabetes Control and Complications Trial

*From the Diabetes Control and Complications Trial Research Group**

Purpose: To answer the following questions regarding the effect of intensive diabetes management on retinopathy in insulin-dependent diabetes mellitus (IDDM): (1) Does intensive therapy completely prevent the development of retinopathy? (2) Are some states of retinopathy too advanced to benefit from intensive therapy? (3) Are the retinopathy endpoints in the Diabetes Control and Complications Trial (DCCT) clinically important? and (4) What other factors influence the effectiveness of therapy?

Methods: A total of 1441 patients, ranging in age from 13 and 39 years and with IDDM of 1 to 5 years' duration and no retinopathy at baseline (primary prevention cohort) or with 1 to 15 years' duration and minimal to moderate nonproliferative retinopathy (secondary intervention cohort), were assigned randomly to either intensive or conventional diabetes therapy. Intensive therapy, aimed at achieving glycemic levels as close to the normal range as possible, included three or more daily insulin injections or a continuous subcutaneous insulin infusion, guided by four or more glucose tests daily. Conventional therapy included one or two daily injections. Seven-field stereoscopic fundus photography was performed every 6 months, for a mean follow-up of 6.5 years (range, 4–9 years).

Results: Intensive therapy reduced the risk of any retinopathy (≥1 microaneurysm) developing in the primary prevention cohort (70% of intensive versus 90% of conventional treatment group; $P = 0.002$) by 27%. It reduced the risk of retinopathy developing or progressing to clinically significant degrees by 34% to 76%. Intensive therapy was most effective when initiated early in the course of IDDM. It had a substantial beneficial effect over the entire spectrum of retinopathy studied in the DCCT and, with rare exceptions, in all patient subgroups.

Conclusion: Although intensive therapy does not prevent retinopathy completely, it has a beneficial effect that begins after 3 years of therapy on all levels of retinopathy studied in the DCCT. The reduction in risk observed in the study is translatable directly into reduced need for laser treatment and saved sight. Intensive therapy should form the backbone of any healthcare strategy aimed at reducing the risk of visual loss from diabetic retinopathy. *Ophthalmology 1995;102:647–661*

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The Diabetes Control and Complications Trial (DCCT) has proven that intensive treatment of insulin-dependent

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Reprint requests to DCCT Research Group, Box NDIC/DCCT, Bethesda, MD 20893.

diabetes mellitus (IDDM) using three or more daily injections of insulin or an insulin pump, along with frequent home blood-glucose monitoring, and aimed at keeping blood-glucose levels as close to the nondiabetic range as possible, reduces the rate of progression of diabetes complications when compared with conventional diabetes therapy. Retinopathy was the principal outcome in the DCCT. The previous summary article[1] reported that intensive therapy led to substantial reductions in the risk of retinopathy development in the 726 patients in the primary prevention cohort and the risk of progression in the 715 patients in the secondary intervention cohort. The objectives of this article are to expand the presentation of retinopathy results and provide sufficient detail to allow clinicians and their patients to answer the following four important clinical questions:

1. Does intensive therapy as administered in the DCCT completely prevent the development of retinopathy?
2. Are some stages of retinopathy too advanced to benefit from this therapy?
3. Are the retinopathy endpoints studied in the DCCT clinically important?
4. What other factors influence the effectiveness of treatment?

Methods

Study Design

Two groups of participants were enrolled in the DCCT.[2,3] The primary prevention cohort included 726 patients with 1 to 5 years' duration of IDDM who were free of retinopathy and microalbuminuria at baseline. The secondary intervention cohort included 715 patients with IDDM of 1 to 15 years' duration who had very mild to moderate background retinopathy and an albuminuria level of less than 200 mg/24 hours.

Among the 1441 patients, 11 died during the trial and an additional 32 patients were inactive during some period of the trial. One patient (secondary intervention-conventional therapy) died before any outcome assessments were performed, and thus does not contribute to the analyses herein. Only 8 of 1430 surviving subjects failed to have a final evaluation with fundus photographs at the end of the study. Patients in the primary prevention cohort were followed for an average of 6.0 years, and those in the secondary intervention cohort were followed for 7.0 years. The duration of participation of each patient varied from 4 to 9 years (total study duration, >9000 patient-years). In June 1993, the trial was stopped, 1 year ahead of the planned termination, by the independent NIDDK-appointed oversight committees.

Treatment Regimens

The randomly assigned treatment regimens were conventional treatment or intensive treatment. Each participant was assigned to a treatment team consisting of nurse, physician, and dietitian. In some centers, regular meetings with a mental health professional were incorporated into the treatment plan. Both treatment regimens and goals have been described in detail elsewhere.[2,4]

The primary goals for the conventional treatment group were the absence of symptoms attributable to hyperglycemia or hypoglycemia, absence of ketonuria, and maintenance of normal growth and development. No predefined targets for glucose control were set. The investigator and patient were masked to HbA1c results, unless values exceeded 13.1%. If this occurred, therapy was adjusted and the HbA1c level was measured monthly until it was less than 13.1%. Up to two injections daily of any mixture of short-acting, intermediate, or long-acting pork, beef/pork, or human insulin and daily urine or blood glucose monitoring were prescribed. Patients were seen by a physician and research nurse every 3 months and by a dietitian usually every 6 months.

The goal of intensive treatment was to achieve and maintain glycemic control as near normal as possible while avoiding severe hypoglycemia. Glycemic goals were as follows: preprandial, 70 to 120 mg/dl; postprandial, less than 180 mg/dl (90–120 minutes after a meal); and at 3:00 AM, 65 to 120 mg/dl. The goal for the HbA1c level was within two standard deviations of the mean for a sample of people without diabetes (<6.05%). Insulin was administered by continuous subcutaneous insulin infusion pump or by multiple (≥3) daily injections. The choice of insulin and methods of delivery rested with the treatment team and participant. Either continuous subcutaneous insulin infusion or multiple daily injections could be tried first with the alternate method used if treatment goals were not met or because of patient preference. Patients in the intensive group were instructed to perform blood glucose tests a minimum of four times daily (preprandial and at bedtime). They also were instructed to test a 3:00-AM sample once weekly with a repeated test the next night if the value was less than 65 mg/dl.

Patients undergoing intensive treatment were hospitalized, generally for 2 to 4 days, to teach them how to administer intensive treatment and develop individual treatment algorithms. Subsequently, patients were seen weekly until they could adequately implement the treatment regimen. They then were seen at least monthly. Telephone contacts were made frequently, as often as daily for the first week, and then weekly thereafter. As reported previously, adherence to assigned treatment exceeded 97%, expressed as percent of study time spent on assigned therapy, for both treatment groups.[1]

Fundus Photography

Seven-field stereoscopic color fundus photographs were taken by certified photographers every 6 months and were graded (masked to treatment) centrally using the Early Treatment Diabetic Retinopathy Study (ETDRS) protocol,[5] which provides a grade for the severity of each lesion of diabetic retinopathy for each eye. Grades for the various lesions were used to derive overall retinopathy severity

levels for each patient according to the ETDRS interim and final scales.[6] The appendix provides abbreviated summaries of both scales (Appendix Tables A1–A3). At the time the DCCT was initiated, only the interim scale was available. During the DCCT, the ETDRS final scale was published, and was shown by the ETDRS to be superior in predicting progression to proliferative retinopathy. In this article, overall results are presented using both scales, but subgroup analyses use only the ETDRS final scale, which is more precise in estimating treatment effects. There are no substantive differences in the analyses using the interim scale and final scale.

The principal outcome specified in the protocol for the primary prevention cohort was the presence of at least one microaneurysm in either eye (at least level 20/<20) at two consecutive 6-monthly gradings. The principal outcome specified for the secondary intervention cohort was a three or more step progression of retinopathy (e.g., from step 3 [20/20] at baseline to step 6 [43/<43] or higher (see Table A2). The presence and severity of macular edema also was assessed from fundus photographs, using ETDRS definitions.[7] Macular edema was defined as retinal thickening and/or obvious hard exudates within 1 disc diameter (1500 μm) of the center of the macula. Clinically significant macular edema was defined as retinal thickening (or hard exudates adjacent to retinal thickening) located within 500 μm of the center of the macula, or an area of retinal thickening at least 1 disc area in size some of which was within 1 disc diameter of the center of the macula.

Subjects and investigators were masked to outcome results unless specified alert levels that might require intervention or closer clinical follow-up were reached. On the interim ETDRS scale, levels 50 and 55 correspond to severe nonproliferative diabetic retinopathy. Subjects and their physicians were unmasked to the severity of retinopathy at level 55 or higher.

Fluorescein Angiography

Stereoscopic fluorescein angiograms were taken and graded using the ETDRS protocol,[8] with modifications of the grading protocol to emphasize recognition of microaneurysms.[9]

Statistical Methods

Event rates are presented as number per 100 patient years based on the ratio of the observed number of patients experiencing the event (cases) to the total patient-years of exposure (at risk). The life-table method was used to estimate the cumulative incidence of events[10] with adjustments for periodically timed assessments.[11] The average relative risk comparing the two treatment groups within each of the primary and the secondary cohorts over the complete period of observation was estimated by a proportional hazards analysis,[10] with adjustment for the baseline grade of retinopathy, and used to test the difference among cumulative incidence curves. The adjusted percentage change in risk for intensive therapy versus conventional therapy was calculated from the average adjusted relative risk of intensive versus conventional treatment as relative risk − 1 multiplied by 100.

The multivariate analysis of differences in proportions was used to compare the treatment groups with respect to the proportions of subjects with a characteristic evident (prevalence) over time.[12] The overall test of group differences used equal weights for the proportions at periodic evaluations.[13]

The Wilcoxon rank-sum test was used to compare the treatment groups with respect to the distributions of ordinal or numeric variables, and the contingency chi-square test for categorical variables.[14]

All outcomes were analyzed based on original random assignment of each patient. All results nominally significant at $P < 0.05$ (two-sided) are indicated.

Results

Table 1 presents a summary of the baseline characteristics of the study cohort. The baseline grade of retinopathy for all patients is presented using the interim and final ETDRS grading scales.[6] For both scales, there is a slight imbalance between treatment groups in the secondary intervention cohort, with a greater fraction of intensively treated patients having the least severe level of retinopathy (20/<20) at baseline. This slight, but statistically significant baseline imbalance is adjusted for in all analyses by stratification for the baseline level of retinopathy.

Primary Prevention Cohort

Development of Retinopathy. Table 2 presents the summary of rates of development of retinopathy and the percent reduction in risk for intensive versus conventional treatment. By life-table analysis, the cumulative incidence of sustained microaneurysms (≥1 microaneurysm in two consecutive 6-monthly sets of photographs) in the intensive treatment group was estimated to be nearly 70%, versus 90% in the conventional treatment group (Fig 1). Both treatment groups had a steady rise in cumulative incidence; the intensively treated group had a relatively lower rate beginning after 3 years of follow-up. Over the 9 years of follow-up, sustained microaneurysms developed at an average rate of 14.9 cases per 100 patient-years in patients who had undergone intensive treatment versus 19.8 per 100 patient-years among patients who received conventional treatment. Thus, intensive treatment slowed the development of retinopathy (average risk reduction over 9 years of 27%; $P = 0.002$), but did not prevent its onset in the majority of patients over the 9-year study period.

Sustained Development of Three-plus Microaneurysms. If the presence of at least three microaneurysms in two consecutive sets of photographs was the minimum requirement to consider the presence of retinopathy, the absolute risk of retinopathy developing was lower in both treatment groups, and the risk reduction with intensive treatment was greater (63%) (Table 2).

Table 1. Baseline Characteristics of the Two Study Cohorts

Characteristics	Primary Prevention Cohort		Secondary Intervention Cohort	
	Conventional (n = 378)	Intensive (n = 348)	Conventional (n = 352)	Intensive (n = 363)
Age (yrs)	26 ± 8*	27 ± 7*	27 ± 7*	27 ± 7*
Men (%)	54	49	54	53
Race (% white)	96	96	97	97
Duration of IDDM (yrs)	2.6 ± 1.4*	2.6 ± 1.4*	8.6 ± 3.7*	8.9 ± 3.8*
Hemoglobin A_{1c} (%)† eligibility	8.8 ± 1.7*	8.8 ± 1.6*	8.9 ± 1.5*	9.0 ± 1.5*
Systolic blood pressure (mmHg)	114 ± 12*	112 ± 11*	116 ± 12*	114 ± 12*
Diastolic blood pressure (mmHg)	72 ± 9*	72 ± 9*	73 ± 9*	73 ± 9*
Retinopathy level, interim scale†§ (%)				
10/10	100	100	0	0
20/<20	0	0	28.7	38.3
20/20	0	0	29.3	29.2
30/<30	0	0	18.2	12.4
30/30	0	0	4.8	5.5
40/<40	0	0	10.8	7.2
40/40	0	0	6.0	4.7
45/<45	0	0	2.0	2.2
45/45	0	0	0.3	0.6
Retinopathy level, final scale†§ (%)				
10/10	100	100	0	0
20/<20	0	0	28.1	38.3
20/20	0	0	29.3	30.0
35/<35	0	0	20.2	13.5
35/35	0	0	11.4	8.8
43/<43	0	0	7.1	5.8
43/43	0	0	1.7	1.4
47/<47	0	0	1.7	1.7
53/<53	0	0	0.3	0

IDDM = insulin-dependent diabetes mellitus.

* Mean ± standard deviation.

† Nondiabetic mean = 5.05 ± 0.5.

‡ See Appendix for definitions.

§ Difference in level of retinopathy at baseline between intensive and conventional treatment groups in the secondary intervention cohort. $P = 0.005$ for the interim scale and $P = 0.02$ for the final scale by Wilcoxon rank-sum test.

Three or More Step Progression. Another outcome defined in the DCCT was the development of a three or more step progression in retinopathy from the baseline level. On both the interim and the final ETDRS scales, three or more step progression constitutes the appearance of microaneurysms plus other lesions. For this outcome, both absolute risks and risk reduction with intensive therapy were similar to those observed for sustained development of three or more microaneurysms (Table 2).

Sustained Three or More Step Progression. An even more conservative definition of retinopathy development is to require an observance of the three or more step progression over two consecutive 6-monthly photographs. Life-table analyses indicated that in 13% of subjects who received intensive treatment, sustained three or more step progression developed over the 9 years of follow-up versus 55% of patients who received conventional treatment, a risk reduction of 76% with intensive therapy (Fig 1).

Diabetes Duration and Treatment Effect. We examined the risk of any retinopathy (at least sustained microaneurysms) and of more severe retinopathy (sustained 3 or more step progression) as a function of IDDM duration (<2.5 years' versus ≥2.5 years' duration) (Table 2). The cumulative incidence in both treatment groups was less among those with shorter versus longer duration, but in both subgroups intensive treatment reduced risk substantially, more so among those with short versus longer duration (Fig 2).

"Pure-primary" Cohort and Treatment Effect. Of the 726 primary prevention patients who were free of retinopathy at baseline based on fundus photographs, 560 also were judged to be free of any retinopathy based on

Table 2. Primary Prevention Cohort: Summary of Absolute Risks and Risk Reductions

	Conventional				Intensive					
	No.	*Cases*	*9-yr Cumulative Incidence (%)*	*Rate per 100 yrs*	*No.*	*Cases*	*9-yr Cumulative Incidence (%)*	*Rate per 100 yrs*	**% Change in Risk***	**(95% CI)**
All Primary Prevention Patients										
Sustained† MA	378	254	89.9	19.8	348	189	69.8	14.9	−27.2	(−40.3, −11.3)
≤2.5-yr duration	214	129	88.9	16.7	182	78	62.1	10.7	−40.0	(−55.2, −19.5)
>2.5-yr duration	164	125	91.8	24.5	166	111	78.3	20.7	−16.5	(−36.4, 9.5)
Sustained 3+ MA	378	150	69.7	8.8	348	63	36.5	3.6	−63.2	(−72.8, −50.3)
3-step progression	378	161	84.9	9.1	348	73	39.0	4.2	−59.8	(−69.8, −46.5)
Sustained 3-step progression	378	91	55.3	4.7	348	23	13.4	1.2	−76.2	(−85.0, −62.3)
≤2.5-yr duration	214	36	49.1	3.3	182	4	6.7	0.4	−89.2	(−96.2, −69.4)
>2.5-yr duration	164	55	61.7	6.6	166	19	20.1	2.1	−69.5	(−82.0, −48.3)
Fluorescein Negative (pure primary)										
Sustained MA	292	193	90.0	19.1	268	140	67.9	14.0	−29.2	(−43.6, −11.0)
Sustained 3-step progression	292	69	55.1	4.6	268	10	6.3	0.7	−86.6	(−93.1, −73.8)

CI = confidence interval; MA = microaneurysms.

* Obtained as 100 × (RR[I:C]−1). Negative values indicate a decrease in risk. Average relative risk RR(I:C) obtained from a proportional hazards model.

† Sustained is defined as presence of the stipulated outcome in at least two consecutive sets of 6-monthly fundus photographs.

fluorescein angiography. The cumulative incidence of sustained microaneurysms and the corresponding risk reductions in this fluorescein-negative "pure-primary" subset of primary prevention patients (Table 2) are nearly identical to those seen in all patients of the primary-prevention cohort. With conventional therapy, the cumulative incidence of sustained three or more step progression within the "pure-primary" cohort is the same as in the total primary prevention cohort. However, the cumulative incidence is less in the intensively treated "pure-

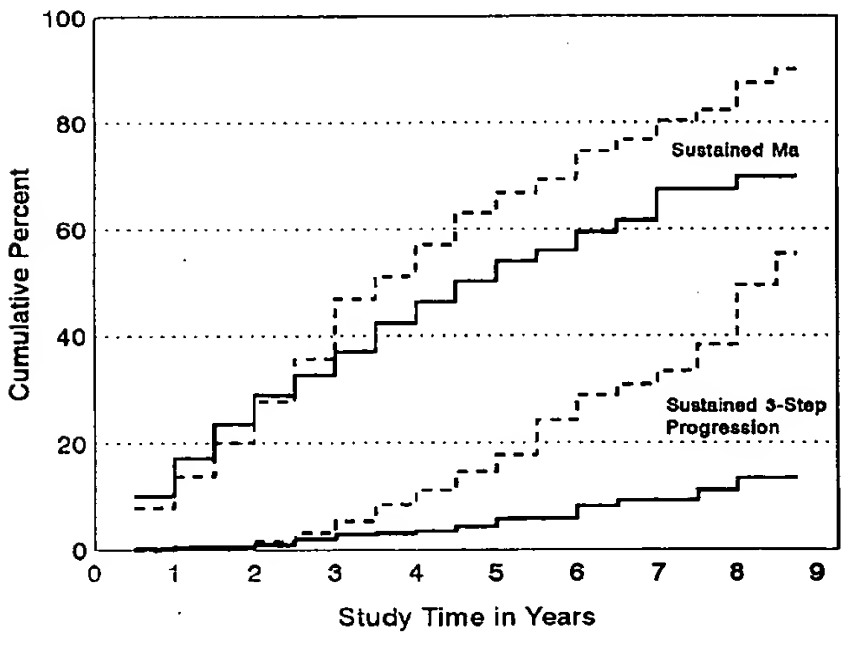


Figure 1. Cumulative incidence plots of one or more sustained microaneurysms (ma) and of sustained three-step or more progression from no retinopathy in the intensive (solid line) and the conventional (interrupted line) treatment groups for the primary prevention cohort. The average adjusted risk reduction for one or more sustained microaneurysms developing with intensive therapy is 27% ($P = 0.002$). The average adjusted risk reduction for sustained three-step or more progression developing with intensive therapy is 76% ($P < 0.001$).

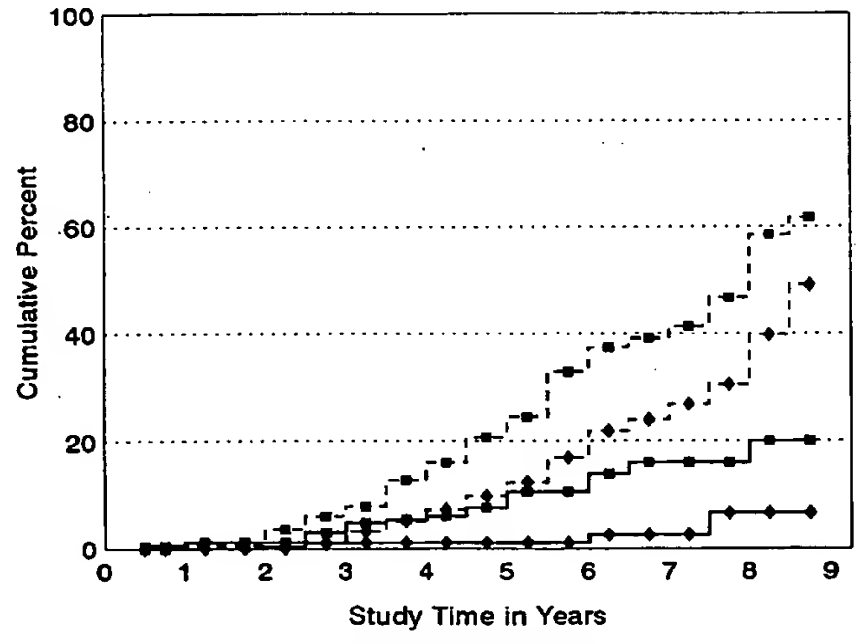


Figure 2. Cumulative incidence plots of sustained three-step or greater progression in the intensive (solid line) and the conventional (interrupted line) treatment groups for the primary prevention cohort with 2.5 or more (closed diamonds) years of insulin-dependent diabetes mellitus duration and for the primary prevention cohort with greater than 2.5 years but less than 5 years of insulin-dependent diabetes mellitus duration (closed squares). The average adjusted risk reduction with intensive therapy in the short duration group is 89% ($P < 0.001$). The average adjusted risk reduction with intensive therapy in the 2.5- to 5-year duration group is 69.5% ($P < 0.001$).

primary" group than in all patients in the primary prevention group, resulting in an 86.6% reduction in risk.

Secondary Intervention Cohort

Table 3 presents a summary of the absolute and relative risks with intensive versus conventional treatment within the secondary intervention cohort.

Three or More Step Progression. The 9-year cumulative incidence of three or more step progression was 56% in patients who received intensive treatment versus 78% in those who were treated conventionally, a risk reduction of 34% with intensive treatment (Fig 3A) (Table 3). With the final ETDRS scale, numbers and rates of events were lower, and the risk reduction was greater than with the interim scale (Table 3).

Sustained Three or More Step Progression. Table 3 also summarizes the life-table analyses of a sustained three or more step progression using the interim and final ETDRS scales, and the estimated cumulative incidence using the final ETDRS scale is presented in Figure 3B. Using the final scale, the estimated cumulative incidence after 9 years among patients who received intensive treatment was 17%, versus 49% among those who received conventional treatment, with an average risk reduction of 65% (Table 3; Fig 3B). As with the three or more step progression that was not sustained, the incidences using the final scale were slightly less, but the estimated risk reduction was slightly greater, than was the case with the interim scale.

Severe Nonproliferative Diabetic Retinopathy. The cumulative incidence of severe nonproliferative diabetic retinopathy (level 55 or worse), which was a stipulated alert level, is presented in Figure 3A. The 9-year rates were 9.2% for patients who received intensive treatment and 26% for those who received conventional treatment, with an average risk reduction of 47%. The risk reduction of severe nonproliferative diabetic retinopathy with intensive therapy, using the final ETDRS scale instead of the DCCT alert, was 61%, somewhat greater than the risk reduction using the DCCT alert level (Table 3).

New Vessels on the Disc or Elsewhere. New vessels on the disc or elsewhere are among the most serious vision-threatening lesions of diabetic retinopathy. Of the 46 conventional treatment patients who developed new vessels, 45 patients developed new vessels elsewhere, and in 19 new vessels developed on the disc (Table 3). Thus, 18 of the 19 patients who developed new vessels on the disc, the more advanced of the two lesions, also developed new vessels elsewhere. Among the patients who received intensive treatment, new vessels developed in 22, with new vessels elsewhere in 22 and new vessels on the disc in 6, all of whom also had new vessels elsewhere. The 9-year incidence rates for new vessels on the disc and/or new vessels elsewhere were 24% and 8%, respectively, for patients who received conventional and intensive treatment, with a risk reduction of 48% with intensive therapy (Fig 3C).

Macular Edema and Clinically Significant Macular Edema

Macular edema and clinically significant macular edema, which is vision threatening, were categorized separately by the ETDRS.[7] Nearly twice as many cases of macular edema as clinically significant macular edema were observed in each treatment group. For macular edema, the cumulative incidence over 9 years was estimated to be 27% among patients who received intensive treatment, versus 44% among those who received conventional treatment, with a risk reduction of 29% ($P = 0.025$). The cumulative incidence of clinically significant macular edema was estimated to be 15% and 27% in the two groups, respectively, with a risk reduction of 23%, the latter not statistically significant.

Any Laser Therapy. Panretinal photocoagulation was recommended for high-risk proliferative diabetic retinopathy, as was consideration of focal photocoagulation for clinically significant macular edema. By life-table analysis over the 9 years of follow-up, an estimated 7.9% of subjects who received intensive treatment would require at least one episode of laser treatment, versus approximately 30% of those who received conventional treatment, with a risk reduction of 59% ($P = 0.001$; Table 3).

Laser Therapy and Loss of Vision

None of the patients in the primary prevention cohort had loss of vision (visual acuity, 20/200 or worse). In the secondary intervention cohort, three patients who received conventional treatment, but no patients who received intensive treatment had loss of vision. In the primary prevention cohort, only four patients were treated with photocoagulation (2 in each group) for either high-risk characteristics or clinically significant macular edema. In the secondary intervention cohort, 5.5% of patients in the intensively treated group versus 14.2% of patients in the conventionally treated group received laser treatment. In both treatment groups, a slightly higher proportion of eyes received focal photocoagulation for clinically significant macular edema than panretinal photocoagulation for proliferative retinopathy, and a small fraction of eyes received both types of treatment. In each case, the percentage of eyes treated with photocoagulation in the intensively treated group was less than half that within the conventionally treated group.

Retinopathy Status at Final Visit

Life-table analyses of cumulative incidence do not consider fluctuations in the level of retinopathy over time in individual subjects and do not reflect subsequent further progression, or regression, after the initial event is reached. An alternate approach is to consider the retinopathy status of all patients at their last visit during the study stratified by the length of follow-up (the longest follow-up was 9 years). We summarize these data by considering the prevalence of either no worsening or worsening by three or

Table 3. Secondary Intervention Cohort: Summary of Absolute Risks and Risk Reductions

	Conventional				Intensive					
	No.	Cases	9-yr Cumulative Incidence (%)	Rate per 100 yrs	No.	Cases	9-yr Cumulative Incidence (%)	Rate per 100 yrs	% Change in Risk*	(95% CI)
3-step progression										
Interim	351	209	77.7	12.9	363	166	56.3	9.2	−33.5	(−46.3, −17.7)
Final†	351	165	68.6	9.0	363	110	40.2	5.5	−43.0	(−55.6, −26.9)
Sustained 3-step progression										
Interim	351	143	54.4	7.8	363	77	26.5	3.7	−54.1	(−65.5, −39.0)
Final†	351	116	49.2	5.9	363	48	17.1	2.2	−64.5	(−74.8, −49.8)
DCCT alert level (55/<55)	351	52	26.1	2.4	363	26	9.2	1.1	−46.8	(−67.2, −13.7)
SNPDR (53/<53+), final scale†	351	68	32.1	3.2	363	26	9.0	1.1	−60.8	(−75.3, −37.7)
NVD	351	19	7.6	0.8	363	6	2.4	0.3	−64.3	(−85.9, −9.8)
NVE	351	45	23.4	2.1	363	22	8.1	0.9	−46.7	(−68.4, −10.3)
NVD/and or NVE	351	46	23.6	2.1	363	22	8.1	0.9	−47.9	(−69.0, −12.5)
Macular edema	351	113	44.3	5.8	363	77	26.7	3.6	−29.2	(−47.7, −4.3)
Clinically significant macular edema	351	63	27.4	3.0	363	45	15.3	2.0	−23.3	(−48.1, 13.3)
Laser treatment	351	50	29.8	2.3	363	20	7.9	0.8	−58.8	(−75.8, −30.0)

CI = confidence interval; DCCT = Diabetes Control and Complications Trial; SNPDR = severe nonproliferative diabetic retinopathy; NVD = new vessels on or within 1 disc diameter; NVE = new vessels elsewhere.

* % obtained as 100 × (RR[I:C]−1). Negative values indicate a decrease in risk. Average relative risk RR(I:C) obtained from a proportional hazards model, adjusted for baseline retinopathy level. Analyses so adjusted used stratification by the corresponding scale (interim versus final), with the highest categories combined: levels 45/<45 and above for the interim scale, levels 47/<47 and above for the final scale.

† Risk reduction adjusted for baseline level of retinopathy using the final scale.

more steps compared with baseline. Each is expressed as a simple percentage of patients (Fig 4 and Table 4).

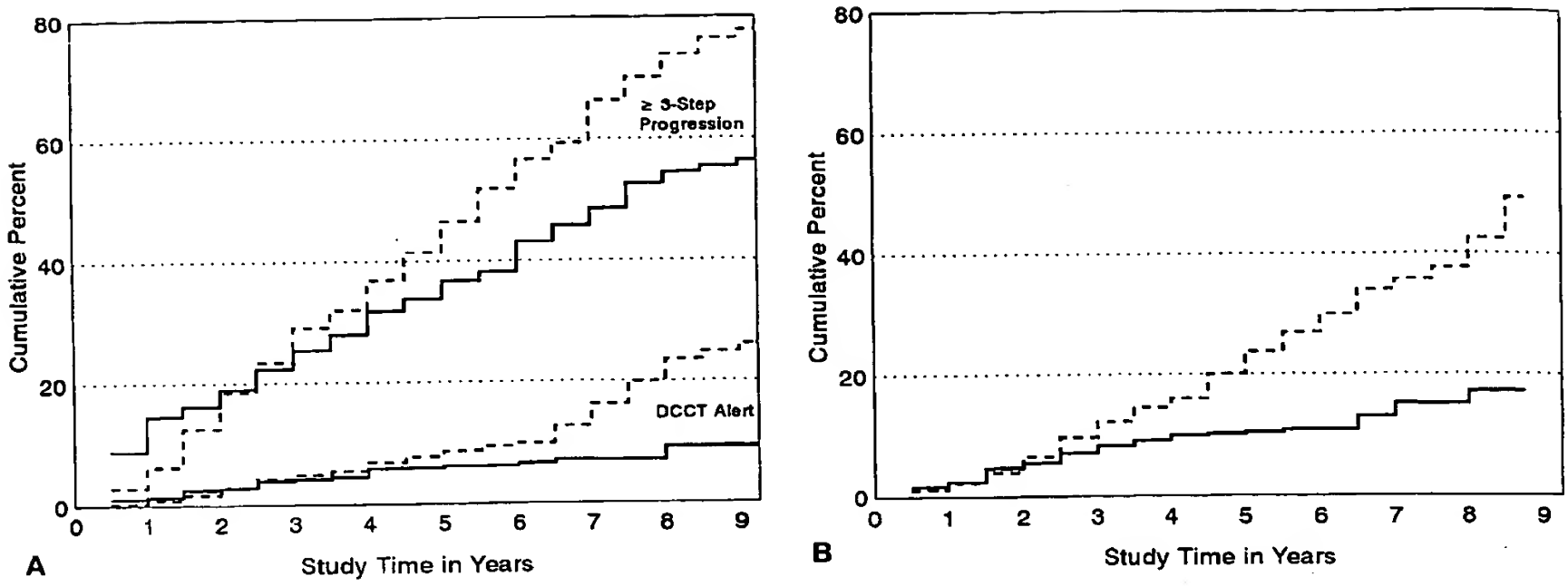
In the primary prevention cohort (Fig 4A), the proportion with no worsening (lower bars) declined over time in both treatment groups, reflecting progression of retinopathy. In the intensively treated group at 5 years, 51% of patients were still no worse than at baseline, versus 33% among the conventionally treated group. This difference of approximately 20% is maintained in subsequent years. In the secondary intervention cohort (Fig 4B) at 5 years, 48% of the intensively treated group was no worse than at baseline, compared with 32% of the conventionally treated group. In subsequent years, differences between intensive and conventional therapy persist. In both figures, the prevalence of retinopathy worse by three or more steps (upper bars) is markedly less in the intensively treated group than in the conventionally treated group, especially after 5 or more years of follow-up.

Table 4 presents an overall summary of the prevalence of these findings for all final visits combined. The mean of the prevalence of three-or-more-steps worsening among the patients who received intensive treatment over all final visits is 82% less than that for those who received conventional treatment in the primary cohort and 60% less in the secondary cohort ($P < 0.001$ for each cohort). Similarly, the probability of no worsening is on average nearly 100% higher among intensively versus conventionally treated groups in the primary cohort, and is approximately 50% higher in the secondary cohort ($P < 0.001$ in each cohort).

Entry Level of Retinopathy and Treatment Effect within the Secondary Intervention Cohort

Analyses of cumulative incidence and prevalence were conducted within subgroups of the population defined according to the baseline level of retinopathy within the secondary intervention cohort. In life-table analyses of cumulative incidence of a sustained three or more step progression, the three subgroups with the mildest degree of retinopathy at baseline (20/<20, 20/20, and 35/≤35) each showed a substantial treatment effect, ranging from 61% to 79% reduction in risk for intensive versus conventional treatment (Table 5). Among the 70 patients who entered with moderate nonproliferative diabetic retinopathy (level 43 or worse), 31 (14 intensive, 17 conventional) had three or more steps of progression. However, 20 of these patients (10 in each group) progressed within the first 3 years, a period during which little beneficial treatment effect was manifest. Forty-four percent of patients who had intensive treatment and 63% of those who had conventional treatment were estimated to have a sustained three or more step progression within 9 years.

In life-table analyses of progression to level 53 (severe nonproliferative diabetic retinopathy), the three subgroups with the least retinoplasty at entry had substantial risk



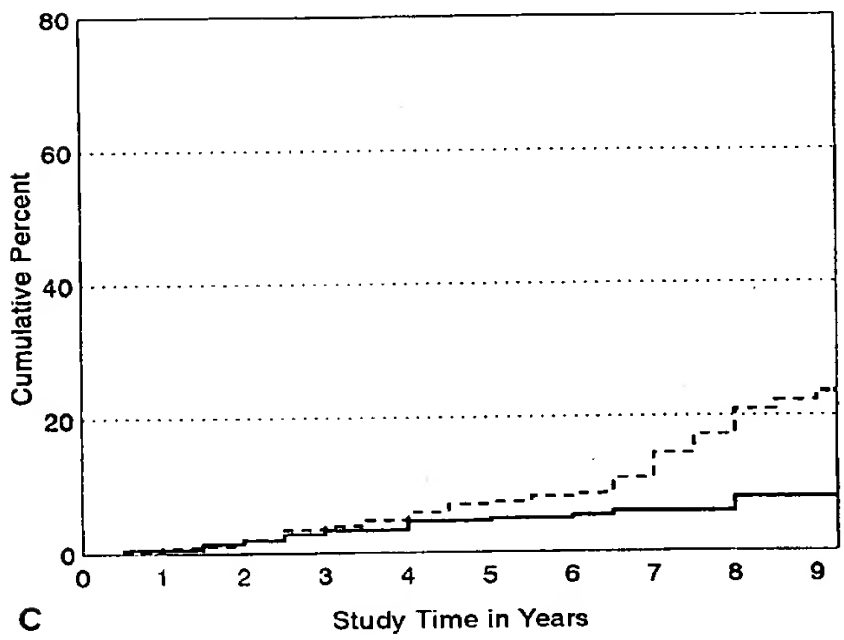


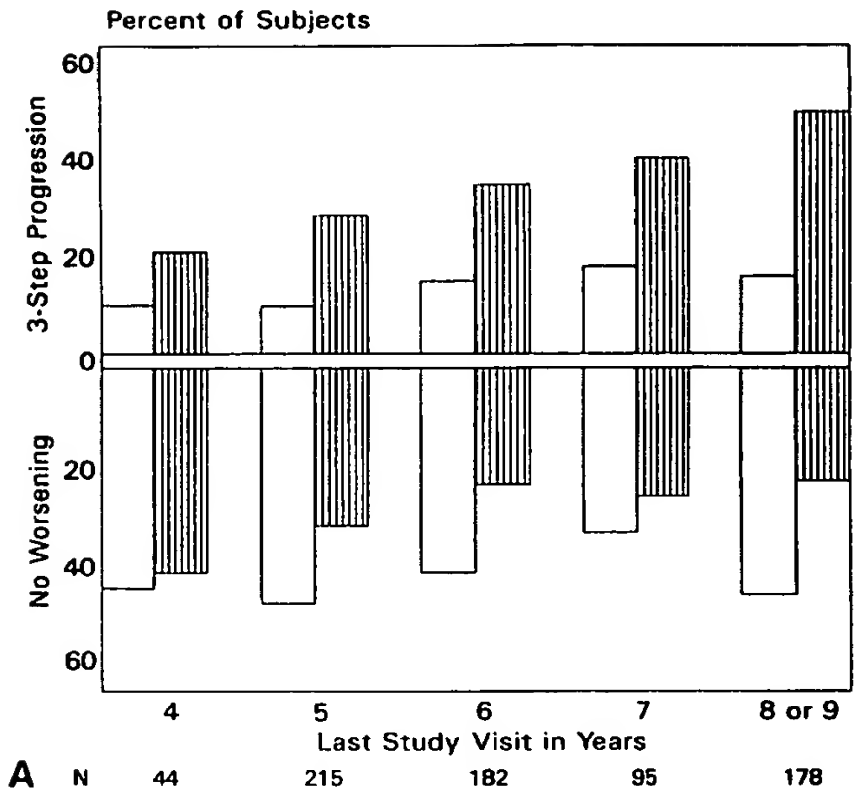
Figure 3. A, cumulative incidence plots of three-step or greater progression from the baseline level of retinopathy and of severe nonproliferative diabetic retinopathy (SNPDR—DCCT Alert) in the intensive (solid line) and the conventional (interrupted line) treatment groups for the secondary intervention cohort. Using the interim Early Treatment Diabetic Retinopathy Study scale, the average adjusted risk reduction of a three-step or greater progression developing with intensive therapy is 34% ($P < 0.001$). The average adjusted risk reduction of developed SNPDR with intensive therapy is 47% ($P = 0.011$). B, cumulative incidence of sustained three-step or greater progression from the baseline level of retinopathy in the intensive (solid line) and the conventional (interrupted line) treatment groups for the secondary intervention cohort using the final Early Treatment Diabetic Retinopathy Study scale. The average adjusted risk reduction associated with intensive therapy is 65% ($P < 0.001$). C, cumulative incidence of neovascularization of the disc and/or neovascularization elsewhere in the intensive (solid line) and the conventional (interrupted line) treatment groups for the secondary intervention cohort. The average adjusted risk reduction association with intensive therapy is 48% ($P = 0.014$).

reductions, ranging from 71% to 88% with intensive therapy (Table 5). Patients at level 43 or worse at baseline had only a 14% reduction in risk with intensive therapy, substantially less than that seen in the other subgroups. Among these 70 patients, 37 progressed to severe nonproliferative diabetic retinopathy (16 in the intensive treatment group, 21 in the conventional treatment group) and usually within the first 3 years (14 in the intensive treatment group, 13 in the conventional treatment group).

Table 6 describes the treatment group differences at the final visit in the prevalence of at least three steps worse and of no worsening within the four subgroups based on baseline retinopathy level. Patients who entered with the most severe levels of retinopathy (level 43 or worse) demonstrated treatment group differences in mean prevalence that were comparable to those seen in the other subgroups and in the total cohort. Although patients in level 43 or worse showed rapid progression during the first 3 years in both treatment groups, the level of retinopathy among patients who had intensive treatment stabilized thereafter, whereas the level of retinopathy in the patients who had conventional treatment continued to worsen (see ref. 15 for detailed results). In this subgroup, the mean prevalence of patients who had intensive treatment who were no worse at the final visit than at baseline was 43%, versus 25% among those who had conventional treatment, with a corresponding mean 99% increase in the prevalence of this favorable outcome.

Other Subgroup Analyses

To assess whether the treatment effects obtained in the entire study cohort applied similarly to different segments of the population of patients with IDDM, analyses also were conducted within subgroups of patients defined on the basis of selected baseline characteristics for the primary prevention and secondary intervention cohorts (Table 7). Because the sample size was reduced greatly within each subgroup, treatment effects within subgroups were not expected to reach statistical significance by the usual cri-



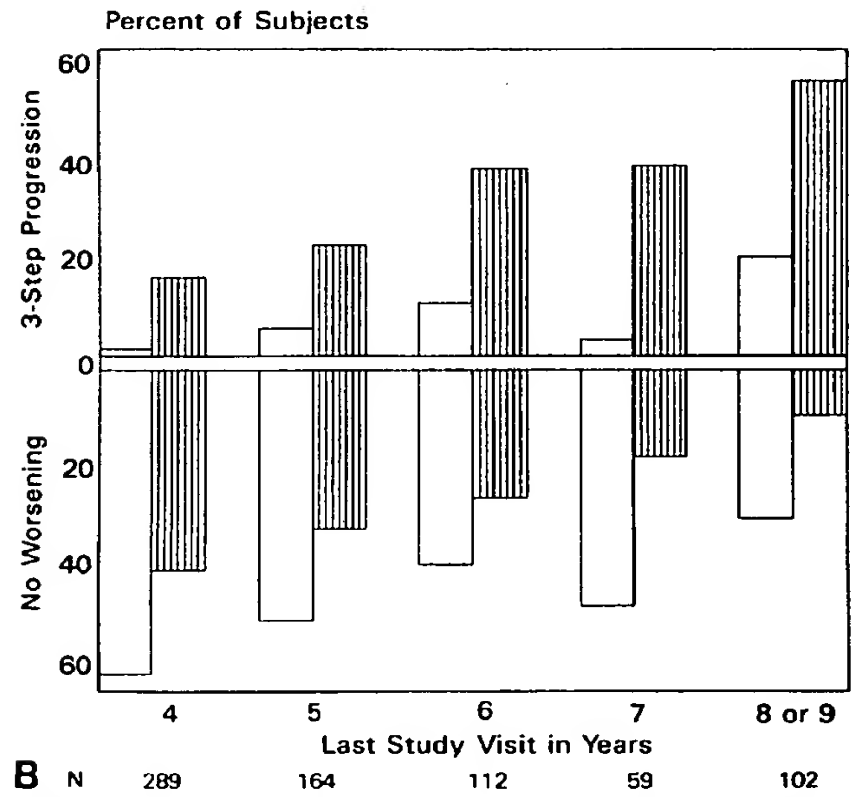


Figure 4. A, prevalence of subjects with no worsening (lower segment) and the prevalence of subjects with worsening by three or more steps (upper segment) in the intensive (white histogram) and the conventional (vertical line histogram) treatment groups in the primary prevention cohort at the final study follow-up visit held in the specified year. N = the total number of patients whose last visit was during the indicated year of follow-up. B, same for subjects in the secondary intervention cohort.

teria. Therefore, to assess whether significant differences in treatment effect existed between subgroups, a test of heterogeneity of treatment effect was performed.

In addition to the variables listed in Table 7, we analyzed the influence of sex, age (adolescents versus adults), tertiles of mean blood pressure, quartiles of daily insulin dose, quartiles of albumin excretion rate, and quartiles of total serum cholesterol, calculated low-density cholesterol, and high-density cholesterol levels on the treatment effect. Data for all subgroups with $P < 0.05$ for heterogeneity of treatment effect in either cohort and data for quartiles of screening HbA1c are shown in Table 7. In the primary prevention cohort, the beneficial effect of intensive therapy was evident in all subgroups, except in the very small group of patients with neuropathy. In the secondary intervention cohort, a beneficial effect was present in the somewhat larger subgroup with neuropathy, although the effect tended to be insignificantly smaller than in patients without neuropathy in the secondary intervention group at baseline. In the secondary cohort, there appeared to be no beneficial treatment effect for patients in the lowest triglyceride quartile ($P = 0.01$), but in the primary cohort the trend was in the opposite direction, with somewhat less treatment effect in the highest quartile ($P = 0.06$; Table 7). In the secondary cohort, treatment effect appeared to be somewhat less for patients in the highest quartile of glomerular filtration rate ($P = 0.03$), but no such trend was apparent in the primary cohort. Rates of sustained progression increased with increasing baseline HbA1c in both treatment groups in both cohorts, but treatment effect remained fairly constant. In all other defined subgroups, a beneficial effect of intensive treatment was evident.

Discussion

Previous epidemiologic studies[16] and a clinical trial[17] have demonstrated an association between glycemia and retinopathy and a beneficial effect of intensive therapy on retinopathy, respectively. The consistent, large beneficial effect of intensive therapy in delaying onset and/or slowing progression of retinopathy, nephropathy and neuropathy in the DCCT resulted in the recommendation to implement intensive therapy in most patients with IDDM. The detailed analyses of retinopathy presented here provide answers to four clinically relevant questions that place the DCCT results in perspective.

1. Does intensive therapy as performed in the DCCT completely prevent the development of retinopathy? Table 1 and Figure 1 show that 70% of patients who had intensive treatment had sustained microaneurysms, even if they did not have retinopathy on fluorescein angiography at baseline. Although intensive therapy reduced the development of the first visible signs of retinopathy by 27%, it could not completely prevent retinopathy from developing. However, using a more conservative outcome of at least three microaneurysms at two consecutive visits, the size of the risk reduction with intensive treatment increased to 63%. If an even more conservative outcome of sustained three or more step progression is used, intensive therapy achieved a 90% risk reduction in patients with 2.5 years of diabetes or less, or

Table 4. Prevalence within Baseline Retinopathy Strata at the Last Visit Using the Final Early Treatment Diabetic Retinopathy Study Scale*

Baseline Retinopathy Strata	Mean Prevalence (risk)		Mean Change in Risk	
	Conventional	*Intensive*	%	*(95% CI)*
At Least 3 Steps Worse				
Primary cohort	34.7	8.3	−82.2	(−89.9, −68.6)
Secondary cohort	34.6	13.7	−60.1	(−73.0, −40.9)
No Worsening				
Primary cohort	25.5	46.6	99.9	(49.4, 167.4)
Secondary cohort	29.2	42.8	49.6	(18.3, 89.1)

CI = confidence interval.

* Simple unweighted mean of prevalence and of % change in risk over final visit year strata 1–4 (combined), 5, 6, 7, and 8–9 (combined).

in patients with normal fluorescein angiograms at baseline. Intensive therapy is more effective when initiated early in the course of IDDM.

2. Are some stages of retinopathy too advanced to benefit from intensive therapy? An analysis of cumulative incidence (Table 5) suggests that eyes with more advanced retinopathy (level 43/<43 or worse) failed to benefit, or had a decreased benefit, from intensive therapy. In contrast, when the results of the final evaluations of all patients were analyzed, the chance of no worsening was increased by intensive treatment in this subgroup as it was in the milder subgroups (Table 6). This is a reflection of the greater likelihood of recovery from a three or more step progression among patients receiving intensive therapy.[15] Although all levels of retinopathy included in the study appeared to benefit from intensive therapy, intensive therapy is more effective in preventing progression when initiated early in the course of IDDM. Whether patients with more advanced retinopathy than included in this study would benefit from intensive therapy is unknown. The disease process appears to have considerable momentum as witnessed by the several years of intensive therapy needed before a treatment effect becomes manifest. It is unlikely that intensive therapy alone can halt the progression of advanced retinopathy. Careful ophthalmic monitoring is essential in patients with more advanced retinopathy, who may require laser photocoagulation.

Table 5. Secondary Intervention Cohort: Cumulative Incidence within Baseline Retinopathy Strata (final scale)

	Conventional				Intensive					
	No.	*Cases*	*9-yr Cumulative Incidence (%)*	*Rate Per 100 yrs*	*No.*	*Cases*	*9-yr Cumulative Incidence (%)*	*Rate Per 100 yrs*	**% Change in Risk***	*(95% CI)*
Sustained 3-step Progression										
Baseline retinopathy strata										
20/<20	100	24	38.4	4.2	140	16	18.2	1.8	−61.4	(−79.7, −26.4)
20/20	103	39	54.3	6.6	109	10	11.5	1.5	−79.1	(−89.6, −57.9)
35/≤35	110	36	45.5	5.9	82	8	11.8	1.6	−76.2	(−89.1, −48.4)
43/<43+	38	17	63.4	9.4	32	14	43.8	9.5	3.6	(−49.9, 114.1)
Severe NPDR (level 53/<53+)										
Baseline retinopathy strata										
20/<20	100	5	8.9	0.8	140	1	2.6	0.1	−88.2	(−98.6, 2.0)
20/20	103	13	22.3	1.9	109	4	3.7	0.6	−70.6	(−90.5, −9.6)
35/≤35	110	29	47.3	4.4	82	5	10.3	1.0	−80.7	(−92.6, −49.7)
43/<43+	38	21	67.4	12.8	32	16	54.5	11.3	−14.1	(−56.4, 69.0)

CI = confidence interval; NPDR = nonproliferative diabetic retinopathy.

* % obtained as 100× (RR[I:C]−1). Negative values indicate a decrease in risk. Average relative risk RR(I:C) obtained from a proportional hazards model.

Table 6. Secondary Intervention Cohort: Prevalence within Baseline Retinopathy Strata at the Final Visit Using the Final Early Treatment Diabetic Retinopathy Study Scale*

	Mean Prevalence (risk)		Mean Change in Risk	
	Conventional	*Intensive*	%	*(95% CI)*
At Least 3 Steps Worse				
Baseline Retinopathy Strata				
20/<20	32.2	12.6	−66.4	(−82.4, −36.0)
20/20	34.0	9.9	−75.1	(−88.3, −47.1)
35/≤35	33.7	13.0	−62.4	(−81.1, −24.9)
43/<43+	50.4	30.6	−38.5	(−68.3, 19.1)
No Worsening				
Baseline Retinopathy Strata				
20/<20	20.5	34.1	91.2	(7.7, 239.5)
20/20	23.6	42.4	79.8	(18.2, 173.4)
35/≤35	40.6	62.9	58.4	(18.4, 112.9)
43/<43+	25.3	42.8	98.5	(−8.3, 329.6)

CI = confidence interval.

* Simple unweighted mean of prevalence and of % change in risk over final visit year strata 1-4 (combined), 5, 6, 7, and 8-9 (combined).

3. Are the principal study endpoints stipulated by the DCCT clinically important? A wide spectrum of retinopathy endpoints was analyzed. The most severe, development of clinically significant macular edema, severe nonproliferative diabetic retinopathy, new vessels elsewhere, and new vessels on the disc, all represent clinically important retinopathy with significant risk of visual loss. Prevention of these lesions clearly saves sight, and the DCCT demonstrated 23% to 64% reductions in them with intensive therapy. Looking at less severe endpoints, such as three or more step progression and sustained three or more step progression in both cohorts, and sustained microaneurysms in the primary cohort, a major effect of intensive therapy is observed. In each instance as the specificity of the endpoint increases from less to more severe disease, the number of cases drops (see Tables 2 and 3) as would be expected, but the beneficial effect of intensive treatment (% risk reduction) increases. This finding suggests that the benefit of intensive therapy is measured best by lesions that are less likely to fluctuate and are less sensitive to variations in grading.
4. Are there other factors that influence the effectiveness of treatment? Intensive treatment reduced the risk of development or progression of retinopathy in all subgroups analyzed. Although the small subgroup of patients with neuropathy present at baseline in the primary prevention cohort showed no benefit, treatment effect in the larger subgroup of such patients in the secondary cohort did not differ significantly from that in patients without neuropathy. Similarly, the absence of treatment effect for patients in the lowest triglyceride quartile in the secondary cohort must be considered in the context of an opposite trend in the primary cohort. Because triglycerides are affected significantly by diabetes control and patients who had intensive treatment had a 5- to 10-mg/dl decrease in triglyceride levels during the first year of the study that was maintained, it is possible that these triglyceride effects reflect differences in diabetes control at baseline. We may gain further insight into this interaction when we analyze change in HbA1c and triglyceride levels over time. Patients with high GFR had a smaller treatment effect than those with lower clearances among secondary cohort patients (P = 0.03), but no such relation was seen in the primary cohort. Renal hyperfiltration may reflect retinal hyperfusion, which might accelerate progression of established retinopathy.

All of the analyses indicate that approximately 3 or more years of intensive treatment are required for beneficial effects on retinopathy to become manifest. This may reflect a long-term effect of the previous metabolic state (glycemic history) on the progression of retinopathy. A period of intensive treatment may be required to reverse this process. In addition, early worsening associated with intensive therapy may delay the appearance of a benefit during the first 2 years of treatment. The incidence of early worsening and its prognosis will be the subject of a separate article.

The progression of retinopathy among patients who received intensive treatment appears to be markedly reduced, if not halted, in the long term; in contrast, the rate of progression among patients who received conventional treatment increases with time. This results in increasing benefit with intensive therapy over time. All of the risk reductions presented were average

Table 7. Influence of Selected Baseline Characteristics on the Relative Risk of a Sustained 3-step Progression

Baseline Characteristics	Conventional No.	Conventional Rate (per 100 patient-yrs)	Intensive No.	Intensive Rate (per 100 patient-yrs)	% Change in Risk (95% CI)*	Heterogeneity P†
Neuropathy						
Primary prevention						
No	368	4.525	329	0.848	−83.4 (−90.5, −71.1)	0.018
Yes	8	5.063	17	6.536	39.2 (−73.3, 626.4)	
Secondary intervention						
No	318	5.969	328	2.019	−67.2 (−77.3, −52.5)	0.168
Yes	33	5.685	34	3.646	−39.6 (−77.9, 65.1)	
Triglycerides (mg/dl)						
Primary prevention						
<55	115	3.301	107	0.550	−84.7 (−95.5, −47.8)	0.057
55–70	94	4.115	91	0.802	−81.2 (−93.6, −44.9)	
70–93	105	5.133	80	0.435	−93.3 (−98.4, −71.4)	
≥93	64	6.221	70	2.973	−55.9 (−79.1, −6.7)	
Secondary intervention						
<55	63	2.740	60	2.579	−1.3 (−63.5, 166.5)	0.011
55–70	86	6.009	100	1.577	−76.4 (−88.9, −49.9)	
70–93	88	5.894	91	3.192	−47.1 (−71.3, −2.5)	
≥93	114	7.837	112	1.683	−80.4 (−89.9, −62.0)	
Creatinine Clearance (ml/min/1.73 m^2)						
Primary prevention						
<110	103	4.938	95	1.221	−75.3 (−89.9, −39.4)	0.165
110–126	99	3.498	80	0.474	−87.9 (−97.2, −46.9)	
126–143	89	3.822	90	1.684	−56.0 (−81.0, 1.8)	
≥143	87	5.843	83	0.823	−87.7 (−95.8, −64.4)	
Secondary intervention						
<110	69	6.504	82	2.002	−63.5 (−83.0, −21.6)	0.033
110–126	91	7.843	93	1.802	−79.3 (−89.9, −57.9)	
126–143	93	5.080	95	1.847	−69.6 (−85.7, −35.4)	
≥143	98	4.758	93	2.939	−38.5 (−67.7, 17.1)	
Screening HbA1c						
Primary prevention						
<7.83	110	1.913	109	0.176	−90.8 (−98.8, −28.5)	0.196
7.83–8.82	96	4.138	77	0.238	−94.5 (−99.3, −59.1)	
8.82–10.10	74	4.918	77	1.399	−76.5 (−90.8, −39.8)	
≥10.10	98	7.708	85	2.632	−68.6 (−83.8, −39.1)	
Secondary intervention						
<7.83	79	2.116	62	0.494	−75.6 (−94.7, 13.2)	0.258
7.83–8.82	89	4.331	98	1.951	−55.5 (−78.8, −6.5)	
8.82–10.10	91	4.602	115	1.750	−58.9 (−80.3, −14.3)	
≥10.10	92	13.801	88	4.511	−72.1 (−83.6, −52.5)	

CI = confidence interval.

* % Obtained as 100 × (RR[I:C]−1). Negative values indicate a decrease in risk. Average relative risk RR(I:C) obtained from a proportional hazards model.

† Test of equality of change in risk among categories of the covariate obtained from a test of interaction between treatment groups and the covariate in a proportional hazards model.

risk reductions, reflecting an initial period of 2 or more years in which there is no benefit for intensive treatment, averaged against a period beyond 3 years in which intensive treatment shows increasing reductions in risks.

Although retinopathy was not completely prevented in the study, a 90% treatment effect was demonstrated in patients with short duration of diabetes and in those patients with no retinopathy at baseline. Beyond the profound beneficial effect in very early cases of retinopathy,

intensive therapy had a beneficial effect on all levels of retinopathy included in the study. Even in patients with nonproliferative retinopathy of moderate severity, retinopathy was more likely to regress or remain unchanged over time with intensive therapy. The reduction in risk observed in the study is translatable directly into reduced need for laser treatment and saved sight. Intensive therapy should form the backbone of any healthcare strategy aimed at reducing the risk of visual loss from diabetic retinopathy. Eye care specialists should join other healthcare professionals in educating patients with IDDM about the importance of intensive therapy as a means of improving long-term outcome.

Appendix

Comparison of Early Treatment Diabetic Retinopathy Study Interim and Final Scales

Table A1 provides an abbreviated summary of the Early Treatment Diabetic Retinopathy Study (ETDRS) final scale for individual eyes. Both the ETDRS scale and the interim scale were based on an earlier scale, which had only three steps in the 30 to 50 range (designated levels: 3, 4, and 5). To reflect rough correspondence with the earlier scale, the level designations were expanded to two digits, and to avoid confusion between the interim and final scales, different numbers were used. Thus, levels 35, 43, 47, and 53 in the final scale correspond to levels 30, 41, 45, and 51 plus 55 in the interim scale. Levels 10, 20, and 61 and above essentially are identical in the two scales.

The severity levels for the two eyes of a patient are combined as shown in Table A2 to give a level for the patient. A patient is classified first by the more severely involved eye, with this category divided into two according to the severity level of the second eye. For example, 20/

Table A2. Abbreviated Early Treatment Diabetic Retinopathy Study Scales of Diabetic Retinopathy Severity for Individuals

Final Scale		Interim Scale	
Step	*Level (worse eye/better eye)*	*Step*	*Level (worse eye/better eye)*
1	10/10	1	10/10
2	20/<20	2	20/<20
3	20/20	3	20/20
4	35/<35	4	30/<30
5	35/35	5	30/30
6	43/<43	6	41/<41
7	43/43	7	41/41
8	47/<47	8	45/<45
9	47/47	9	45/45
10	53/<53	10	51/<51
11	53/53	11	51/51
12–23	61/<61 or greater	12	55/<55
		13	55/55
		14–25	61/<61 or greater

Table A1. Abbreviated Summary of the Early Treatment Diabetic Retinopathy Study Final Scale of Diabetic Retinopathy Severity for Individual Eyes

Level	Severity	Definition
10	No retinopathy	Diabetic retinopathy absent
20	Very mild NPDR	Microaneurysms only
35	Mild NPDR	Microaneurysm plus hard exudates, soft exudates (cotton-wool spots) and/or mild retinal hemorrhages
43	Moderate NPDR	Microaneurysms plus mild IRMA or moderate retinal hemorrhages
47	Moderate NPDR	More extensive IRMA, severe retinal hemorrhages, or venous beading in one quadrant only
53	Severe NPDR	Severe retinal hemorrhages in 4 quadrants, or venous beading in at least 2 quadrants, or moderately severe IRMA in at least 1 quadrant
61	Mild PDR	NVE < 1/2 disc area in 1 or more quadrants
65	Moderate PDR	NVE ≥ 1/2 disc area in 1 or more quadrants, or NVD < 1/4–1/3 disc area
71–75	High-risk PDR	NVD ≥ 1/4–1/3 disc area and/or vitreous hemorrhage
81–85	Advanced PDR fundus partially obscured	

NPDR = nonproliferative diabetic retinopathy; PDR = proliferative diabetic retinopathy; IRMA = intraretinal microvascular abnormalities; NVE = new vessels elsewhere; NVD = new vessels on or within 1 disc diameter of optic disc.

Table A3. Percent of Patients in the Early Treatment Diabetic Retinopathy Study in Whom the Eye Assigned to Deferral of Photocoagulation Had Progressed to Proliferative Retinopathy after 1 Year*

Interim Scale			Final Scale		
Definition	*No. at Risk*	*% with PDR after 1 Yr*	*Definition*	*No. at Risk*	*% with PDR after 1 Yr*
Level 30			Level 35		
RH, H/Ma < S/1-3*	23	4.3	RH, H/Ma < M/45	20	5.0
Hard exudate ≥ D/1	153	6.5	Hard exudate ≥ D/1	110	5.5
			Soft exudate ≥ D/1	243	4.1
Level 41			Level 43		
IRMA = D/1-3	58	20.7	H/Ma = M/4-5 to S/1	354	10.5
Soft exudate = D/1-3	481	10.2	IRMA = D/1-3	234	15.0
Level 45			Level 47		
Soft exudate ≥ D/4-5	387	12.7	Both level 43 conditions	323	21.7
IRMA = D/4-5 to M/1	118	32.2	IRMA = D/4-5	99	28.3
Venous beading = D/1	85	32.9	H/Ma = S/2-3	96	30.2
H/Ma = S/1-3	232	23.7	Venous beading = D/1	87	34.5

* Each definition assumes that no more severe definition is met.

† Severity categories for characteristics graded in multiple fields are of the form (maximum severity/extent), where maximum severity can be absent (A), questionable (Q), definitely present (D), moderate (M), severe (S), or very severe (VS), and extent is the number of fields at that severity level. For example, M/2-3 means there are two or three fields from fields 3 to 7 with moderate severity, and none with higher severity.

PDR = proliferative diabetic retinopathy; RH = retinal hemorrhage; H/Ma = retinal hemorrhages and/or microaneurysms; IRMA = intraretinal microvascular abnormalities.

20 designates a patient with microaneurysms only in each eye and 20/<20 a patient with microaneurysms only in one eye and a lesser level in the other (in this case level 10, no retinopathy, is the only lower level, but in other cases, all lower levels are pooled). As shown in Table A2, the final scale has two fewer steps than the interim level, because it has only one level, rather than two, in the 50 to 59 range of the eye scale.

Table A3 provides a detailed comparison of the two scales in the 30 to 40 range, and includes data from the ETDRS on which the final scale was based. The principal change made in the final scale, and the one of greatest importance to the DCCT, was the downgrading of the prognostic importance of soft exudates (cotton-wool spots). To compare the two scales, begin at the bottom of the table and read upward; the risk of proliferative retinopathy after 1 year should decrease as the level decreases. With the interim scale, presence of definite soft exudate in 4 or 5 of the standard photographic fields (or a more severe grade of soft exudate in any 1 field) placed an eye in level 45, and a single soft exudate in any one field placed it in level 41. Note that these two rows seem to be out of order in so far as risk of proliferative retinopathy is concerned. The rates of 10.2% and 12.7% for these rows

Table A4. Lesions Required for Progression of Three Steps or More

Interim Scale		Final Scale	
Baseline Retinopathy Level	*Lesion*	*Baseline Retinopathy Level*	*Lesion*
10/10	SE, one eye (or RH, HE)	10/10	SE, one eye (or RH, HE)
20/<20	SE, one eye	20/<20	SE, both eyes
20/20	SE, one eye	20/20	More severe lesions*
30/<30	SE, each eye	35/<35	More severe lesions*
30/30	SE in all 4 quadrants of one eye (or several in one quadrant)	35/35	More severe lesions*
41/<41 or worse	More severe lesions	43/<43 or worse	More severe lesions*

* Intraretinal microvascular abnormalities, more extensive retinal hemorrhages and/or microaneurysms, or venous beading.

SE = soft exudates (cotton-wool spots); RH = retinal hemorrhage; HE = hard exudate.

reflect the presence of intraretinal microvascular abnormality and/or moderately extensive retinal hemorrhages/microaneurysms accompanying the soft exudates, because when eyes with soft exudates without these accompanying lesions were moved down in the hierarchy (final scale) the progression rate was 4.1%. The final scale was designed on the basis of these data to be ordered, except that soft exudates were left as the highest subdivision of level 35.

Table A4 show the lesions required for progression of three or more steps on each scale for baseline retinopathy subgroups. On the interim scale, development of soft exudates results in progression of three or more steps in any patient free of soft exudates at baseline, but on the final scale this is true only for levels 10/10 and 20/<20 (no microaneurysms or microaneurysms in only 1 eye). The ETDRS interim scale is disadvantageous for DCCT analyses, particularly for those that use cumulative incidence of progression by three or more steps on the scale, because it treats soft exudates as though their association with risk of progression to proliferative retinopathy is stronger than it really is, and because transient development of soft exudates soon after initiation of intensive treatment is a common occurrence.

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Reports

Enhancement of retinal adhesion and subretinal fluid resorption by acetazolamide.

MICHAEL F. MARMOR AND TÖNNIES MAACK.

Intravenous administration of acetazolamide in the rabbit increased the force required to peel retina from the pigment epithelium in vitro and increased the speed with which small experimental nonrhegmatogenous detachments resorbed in vivo. These results suggest that acetazolamide may have clinical application in the management of retinal detachment. (INVEST OPHTHALMOL VIS SCI 23:121-124, 1982.)

Under pathologic conditions the retina may detach from the retinal pigment epithelium (RPE), with a resultant loss of vision. At present, therapy for rhegmatogenous retinal detachment is surgical, with a high rate of anatomic reattachment but only fair success in restoring normal visual acuity.[1] There is no medical therapy available for either rhegmatogenous or nonrhegmatogenous detachments to limit spreading or hasten the resorption of subretinal fluid.

A number of mechanisms interact to keep the normal retina adherent to the RPE.[2] Physical factors include ensheathment of the photoreceptor tips by microvilli from the RPE, viscosity of the intercellular matrix, and fluid pressures within the eye. Metabolic factors are also important, since retinal adhesion is strong in life but falls within minutes of death.[2, 3] The metabolic systems that control retinal adhesion probably reside within the pigment epithelium, which has structural and membrane transport properties similar to those of secretory epithelia elsewhere in the body and thus has the machinery to move fluid in and out of the subretinal space.

In previous work on the rabbit, we have shown that retinal adhesion and subretinal fluid resorption are decreased by respiratory inhibition with cyanide or systemic hypoxia but are increased by the inhibition of active sodium transport with ouabain.[3, 4] Unfortunately, ouabain is systemically toxic and is not likely to have clinical application. Acetazolamide inhibits a different enzyme (carbonic anhydrase) than does ouabain but acts similarly insofar as it blocks the secretion of fluid by various epithelia.[5] The present experiments investigate its effect on retinal adhesion and subretinal fluid resorption. Since acetazolamide is well tolerated systemically, our results suggest that under certain conditions, it might help in the prevention or treatment of retinal detachment.

Methods. All experiments were performed on Dutch rabbits weighing 1.5 to 2 kg. Animals were initially sedated with 12 mg/kg thorazine intramuscularly, followed by 25 mg/kg pentobarbital intravenously, which was augmented as needed to produce full anesthesia. The experiments were performed under ordinary room illumination. For fundus observation the pupil was dilated with 1% cyclopentolate and 10% phenylephrine eyedrops. Two standard physiologic solutions were used: Ames' solution[6] and Hanks' solution (402S; Grand Island Biological, Grand Island, N.Y.). Acetazolamide solutions were prepared from the sodium salt (Lederle Laboratories, Pearl River, N.Y.).

Retinal adhesion was measured, as described previously,[3] by recording the force required to peel retina from the RPE under fluid in vitro. The posterior segment of an enucleated eye was placed immediately in a Petri dish containing oxygenated Ames' solution at 37° and cut into four 5 mm strips, which were studied sequentially at 5 min intervals. Each strip was fixed to a small platform, and the inferior end of the retina was glued with cyanoacrylate to a transducer that measured force as the retina was peeled off. Our previous work has shown that retinal adhesion falls steadily after death, despite maintenance of the tissue in oxygenated Ames' solution. Thus comparison was made only between measurements obtained at the same time after death.

The resorption of subretinal fluid was studied by monitoring the resolution of small experimental detachments (blebs). The blebs were formed in vivo, as described previously,[3] by forcing Hanks' solution into the subretinal space through the tip of a glass micropipette, which entered the eye at the limbus. The limbal hole was not sealed and intraocular pressure was near zero throughout these experiments. Rabbit retina is avascular except within a strip of myelinated nerve fibers, and all blebs were made in avascular areas. Bleb resorption occurred over several hours, primarily by a loss of height rather than diameter. By stereomicroscopic observation at 20 min intervals, the endpoint was estimated when elevation was lost and the pigment epithelial pattern became sharply visible over 50% of the bleb floor.

Results. The effect of acetazolamide on retinal adhesion was first studied by adding the drug to the in vitro bathing solution. Fig. 1, *A*, shows that 10^{-3}M acetazolamide had no significant effect un-

Invest. Ophthalmol. Vis. Sci.
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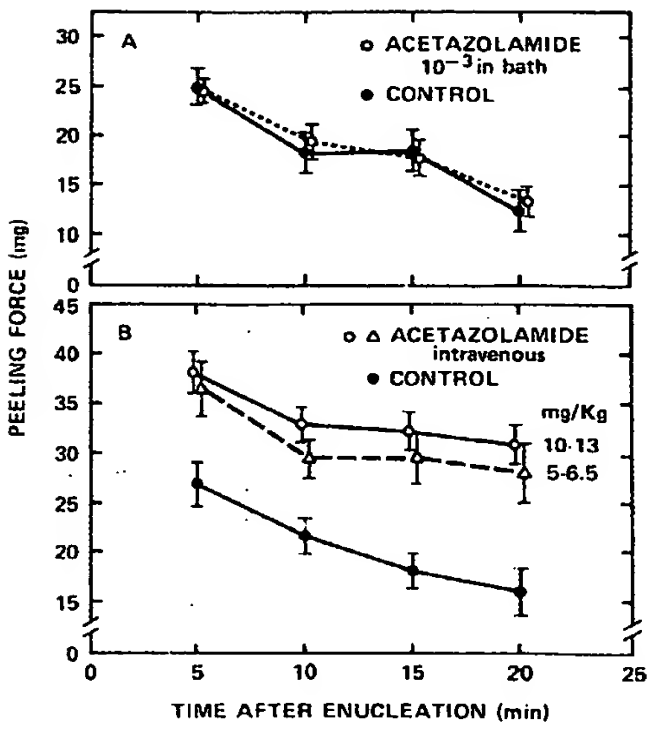


Fig. 1. Retinal adhesion in the rabbit, measured as the force required to peel retina from the RPE, in the presence and absence of acetazolamide. The points represent the average of at least eight measurements, with standard error of the mean shown. A, Effect of acetazolamide in the in vitro bathing solution. B, Effect of systemically administered acetazolamide.

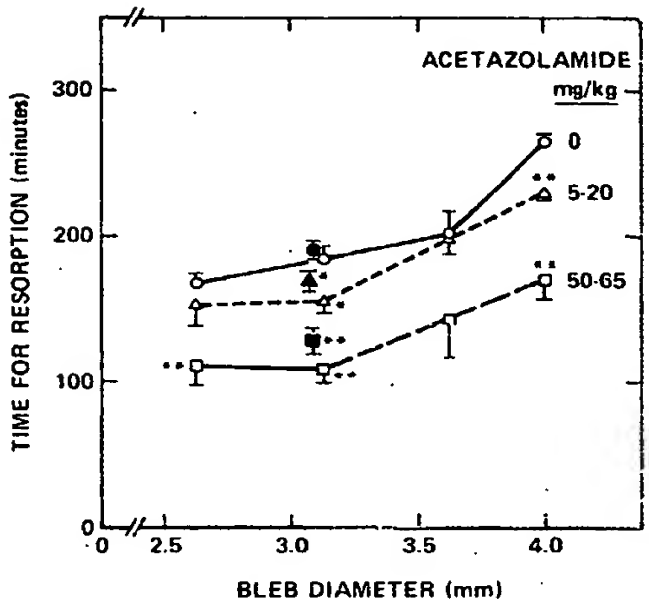
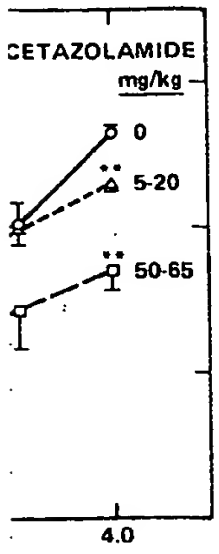


Fig. 2. Average time required for resorption of experimental detachments (blebs) of different sizes, with and without intravenous acetazolamide (at the dosages shown). *Open symbols*, Mean (with standard error) of blebs 2.5 to 2.75, 3.0 to 3.25, 3.50 to 3.75, and 4.0 mm in diameter; *closed symbols*, mean bleb diameter and resorption time (with standard error) for the entire population of data points. Points marked by asterisks differ significantly from the control (*, $p < 0.05$; **, $p < 0.001$).

der these conditions, in contrast to ouabain, which enhances the peeling force when added to the bathing solution.[3] Studies on the frog pigment epithelium indicate that, whereas ouabain-sensitive sodium transport occurs at the apical surface of the cells, active chloride and bicarbonate transport probably occurs at the basal surface, which faces the choroidal blood supply.[7] We postulated that intravenous acetazolamide might reach the basal surface more effectively and allow more time for effects to develop. Fig. 1, *B*, shows the results of adhesion experiments on control eyes and on eyes enucleated approximately 30 min after an intravenous dose of either 5 to 6.5 or 10 to 13 mg/kg acetazolamide (which is comparable by weight to doses used clinically in man for the suppression of aqueous secretion). Acetazolamide markedly increased the peeling force at all intervals after enucleation. The 10 to 13 mg/kg dose appeared to be slightly more effective than the 5 to 6.5 mg/kg dose, but raising the dose to 50 to 65 mg/kg did not increase the peeling force further.

To determine whether systemic acetazolamide would alter the speed of subretinal fluid resorption, we formed small experimental detachments (blebs) shortly after an intravenous dose of acetazolamide and compared them with blebs formed in the absence of drug. We attempted to make these blebs 3.0 to 3.25 mm in diameter, but the size could not be controlled precisely. This made analysis of the data more complicated because large blebs resorb, on the average, more slowly than small ones[4] and bleb diameter had to be taken into account. From 75 eyes there were a total of 67 control blebs, 44 low-dose (5 to 20 mg/kg) blebs, and 22 high-dose (50 to 65 mg/kg) blebs. The low-dose level is comparable by weight to ordinary clinical use of the drug.

Fig. 2 shows the resorption times for blebs grouped at 0.5 mm intervals. The resorption times after acetazolamide were shorter than the control times for all bleb sizes. The time difference was significant (using the t test for unpaired data) at $p < 0.05$ or $p < 0.001$ for many of the points, but



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not all, because of scatter in the data. Fig. 2 also shows the overall population means for bleb diameter and resorption time. Relative to the control time of 190 min, the mean resorption times after acetazolamide were 169 min (low-dose) and 128 min (high-dose). For low-dose acetazolamide this difference represents only an 11% shortening of the resorption time and was barely significant ($p < 0.05$); for high doses the time difference was 33% and highly significant ($p < 0.001$). To ensure that the population means were not biased by the covariance of bleb diameter and resorption time, we also applied a least-squares regression analysis, assuming a common slope for the three groups of data.[8] The regression lines for the acetazolamide data differed significantly from the control line at $p < 0.05$ for low-dose blebs and $p < 0.001$ for high-dose blebs.

In some of these bleb experiments, one eye of an animal served as a control for the other eye, which was studied after acetazolamide. We thought such pairing might reduce the variability of the data, but bleb size could not be exactly matched between the two eyes and the statistical significance of the data was not improved within the small number of experiments performed.

Discussion. Our results show that in the rabbit, retinal adhesion and subretinal fluid resorption can be favorably altered by acetazolamide, a drug that is acceptable for use in man. This suggests that acetazolamide might have applications clinically, possibly to prevent or slow down the spread of detachments (by enhancement of adhesivity) or to hasten the resorption of subretinal fluid. Our bleb resorption model mimics most closely a nonrhegmatogenous detachment such as central serous retinopathy, but adhesion and subretinal fluid resorption are factors whose modification may be of interest in any type of detachment.

We caution strongly that clinical effects are only postulated. We produced a significant effect on adhesion with doses of acetazolamide that are in common clinical use, but these same dose levels had only a marginal effect on resorption. The high doses that enhanced resorption may not be feasible for clinical purposes (although the dose-response curve could be different in primates and man). Furthermore, human retinal disease differs in important respects from the conditions of our rabbit model. Our experimental detachments were nonrhegmatogenous in origin, whereas rhegmatogenous detachments in man are complicated by pathologic changes in the vitreous and traction on the retina. Serous or exudative detachments in man may be complicated by damage to the RPE or by inflammatory disease. Even if acetazolamide proves to be beneficial, its effects may be insufficient to overcome the complicating factors in disease. Finally, and perhaps of greatest importance, we have no evidence as yet that the drug acts similarly in primates; until this can be demonstrated, clinical trials are not warranted.

Acetazolamide blocks the enzyme carbonic anhydrase and inhibits the secretion of both aqueous humor and cerebrospinal fluid.[5] By analogy, it is tempting to ascribe the actions of the drug on adhesion and bleb resorption to inhibition of a transport process. Indeed, the enhancement of adhesion could be explained if acetazolamide facilitated fluid resorption out of the subretinal space (or blocked a transport system that normally moves fluid into the subretinal space), since this would produce a tighter subretinal space and a more viscous intercellular matrix. The most likely site for an effect on transport would be the RPE, which controls ionic movement (and thereby an associated obligate movement of water) to and from the subretinal space. We have shown previously[4] that fluid is unlikely to leave our blebs by other routes such as the subretinal space or across the retinal substance. In the frog, acetazolamide reduces chloride movement across the RPE from retina to choroid, but this flux is in the wrong direction to account for our findings; sodium is transported from choroid to retina but is not affected by acetazolamide.[7] The ionic fluxes across the mammalian RPE have not been as well documented and may not be identical to those in the frog. We have shown previously that inhibition of sodium transport with ouabain can enhance retinal adhesion and subretinal fluid resorption.[3] Studies on the secretion of aqueous and cerebrospinal fluid indicate that sodium transport can be linked to bicarbonate formation[9] and thus may be sensitive to acetazolamide as well as to ouabain. On the other hand, there is still considerable uncertainty as to the localization and significance of carbonic anhydrase in the mammalian RPE.[10, 11]

Acetazolamide could also affect adhesion or fluid movement by less direct means. The drug acts as a diuretic, causing metabolic acidosis and a systemic loss of bicarbonate and potassium. We have shown elsewhere[12] that retinal adhesion in the rabbit is not changed by lowering potassium but falls markedly at low pH. The systemic pH change from a brief diuresis is probably not severe enough to have much effect, and in any event is in the wrong direction to explain our results on adhesion. Intravenous acetazolamide causes an increase in both cerebral and choroidal blood flow,[13] possibly as a

result of systemic and local changes in the chemical and ionic environment. Increased choroidal blood flow might facilitate the resorption of subretinal fluid by serving as a sink for fluid drawn off by ionic or osmotic gradients. A similar result would be achieved if the drug in some way increased the passive permeability of the RPE, although there is no evidence for such an effect.

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Key words: retinal detachment, retinal pigment epithelium, acetazolamide, rhegmatogenous, nonrhegmatogenous, central serous retinopathy

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Autoregulation of the retinal circulation in response to decrease of intraocular pressure below normal.

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The autoregulatory response of the retinal circulation to a short-term reduction in intraocular pressure (IOP) to hypotonic levels was studied in 15 normal subjects by means of the blue-field entoptic phenomenon. This phenomenon allows the perception of the leukocytes flowing in one's own retinal macular capillaries. Subjects were asked to compare the leukocyte speed in one eye with that in the fellow eye while a scleral suction cup was used to raise the IOP in one eye to levels above 25 mm Hg for approximately 12 min. The release of the suction cup caused a drop in IOP to levels between 4 and 7 mm Hg, at which time all subjects reported a higher leukocyte speed (hyperemia) in this eye than in the fellow eye. After an average of 4 min the speed was observed to be equal in both eyes. The average IOP at which the equalization occurred was 6.8 ± 1.3 mm Hg. The retina can therefore normalize leukocyte capillary speed and presumably blood flow at IOPs at least as low as 6.8 mm Hg. The results of 16 experiments on the same eye of one subject suggest that under these experimental conditions, the lowest IOP for which the retina can fully autoregulate is around 6 to 7 mm Hg. (INVEST OPHTHALMOL VIS SCI 23:124-127, 1982.)

Autoregulation, an intrinsic property of many organs, allows a tissue to maintain constant blood flow despite changes in perfusion pressure. A number of researchers[1-6] have investigated the autoregulatory response of the retinal vasculature to decreased perfusion pressure induced by an elevation of intraocular pressure (IOP). Recent

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The Beaver Dam Eye Study

Retinopathy in Adults with Newly Discovered and Previously Diagnosed Diabetes Mellitus

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The prevalence of diabetic retinopathy was examined in people with newly discovered noninsulin-dependent diabetes mellitus (NIDDM) (n = 50) and in those with previously diagnosed diabetes (n = 395) in a population-based study of people between the ages of 43 and 86 years who lived in Beaver Dam, Wisconsin between 1988 and 1990. Retinopathy was determined from stereoscopic fundus photographs. The prevalence of any retinopathy was 10.2% in those with newly diagnosed NIDDM, none had proliferative retinopathy, and 2.0% had macular edema. These data suggest that asymptomatic people discovered to have NIDDM during epidemiologic studies may not need immediate ophthalmoscopic examination at the time of their diagnosis because they have a relatively low risk of danger of visual loss due to diabetic retinopathy at that time. *Ophthalmology 1992; 99:58–62*

Currently, if diabetes is diagnosed after 29 years of age, guidelines recommend that the retina be examined through a dilated pupil to rule out the presence of proliferative retinopathy or macular edema.[1,2] This is based on the observation from some studies that retinopathy may appear at or shortly after the time of diagnosis in people with noninsulin-dependent diabetes mellitus (NIDDM) who comprise the majority of newly diagnosed cases in this age group.[3,4] However, other studies suggest that retinopathy is relatively rare at the time of diagnosis of NIDDM.[5,6] We describe our findings of the prevalence and severity of retinopathy in adults with diabetes, both newly discovered and those with previously diagnosed diabetes (including people who take insulin), who participated in the population-based Beaver Dam Eye Study.

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Materials and Methods

The Beaver Dam Eye Study population has been described in detail in previous reports.[7–9] In brief, a private census of the population of Beaver Dam, Wisconsin was performed from September 15, 1987 to May 4, 1988. Eligibility requirements for entry into the study included living in the city or township of Beaver Dam and being 43 to 84 years of age at the time of the census. Of the 6612 households that were identified by the census, 3715 contained at least 1 person satisfying the age criteria. These households had a total of 5833 individuals between the ages of 43 and 84 years. After completion of the census, 76 additional households with a total of 92 eligible people were identified and are included in the population. Participants were examined over a 30-month period beginning March 1, 1988.

Procedures

Letters describing the study and inviting participation were sent to those subjects who were eligible. The letter was

Table 1. Relationship of Retinopathy Status to Type of Diabetes in the Beaver Dam Eye Study (1988–1990)

Retinopathy Status	Young Onset Insulin-dependent Diabetes (n = 19) (%)	Newly Discovered NIDDM (n = 49) (%)	Previously Diagnosed NIDDM			All Groups (n = 435) (%)
			Using Insulin (n = 79) (%)	*Using Oral Hypoglycemic Agents or Diet (n = 271) (%)*	*Using Insulin and Oral Hypoglycemic Agents (n = 17) (%)*	
Any	68.4	10.2	69.6	29.9	35.3	36.8
Proliferative retinopathy	10.5	0	6.3	0.4	0	1.8
Macular edema	10.5	2.0	10.3	0.7	0	3.0

NIDDM = noninsulin-dependent diabetes mellitus.

followed by a telephone call from the study coordinator, who provided further information about the study and made an appointment for the examination. People who were not interested in participating in the examination were asked to respond (by telephone) to the same questionnaire that was administered at the time of the examination.

Of the 5925 eligible people, 4926 (83.1%) participated in the examination. Two hundred twenty-five people (3.8%) died before the examination, 91 people (1.5%) moved out of the area, and 23 people (0.4%) could not be located. Two hundred sixty-nine people (4.5%) permitted an interview only, and 391 (6.6%) refused to participate. Comparisons between participants and nonparticipants have been presented elsewhere.[9]

The standardized questionnaire administered by the examiners included the questions: "Has a doctor ever said you had diabetes, sugar in your urine, or high blood sugar?" and "How old were you when you learned this?" There were also questions regarding use of diet and oral hypoglycemic agents or insulin for the management of hyperglycemia. Serum glucose was determined using the hexokinase method, and plasma glycosylated hemoglobin was determined using affinity chromatography (Isolab Inc, Akron, OH) for casual blood samples.[10,11]

Stereoscopic 30° color fundus photographs centered on the disc (Diabetic Retinopathy Study [DRS] standard field 1), macula (DRS standard field 2), and a nonstereoscopic color fundus photograph of a modified DRS standard field 3 of each eye were taken.[12] The photographs were mounted in clear plastic sheets. To determine diabetic retinopathy status, all fundus photographs were graded in masked fashion using the modified Airlie House classification scheme.[12–14] Level 10 represents no retinopathy, levels 21 to 51 represent nonproliferative retinopathy of increasing severity, and levels 60 to 80 represent proliferative retinopathy of increasing severity. Retinopathy level for a subject was derived by giving the eye with the higher level greater weight. The presence of clinically significant macula edema was also determined.[15] Diabetic retinopathy could not be graded in ten persons (nine with a previous history of diabetes and one with newly diagnosed diabetes).

Definitions

There were 395 people with a previous history of diabetes mellitus, treated with either insulin, oral hypoglycemic agents, and/or diet. There were 50 people with newly diagnosed diabetes mellitus, defined as no previous medical history of diabetes mellitus or use of hypoglycemic medications for diabetes mellitus and a glycosylated hemoglobin value that was greater than 2 standard deviations above the mean for a given age-sex group (43 to 54 years of age, men >9.5% and women >9.6%; 55 to 64 years of age, men >9.4% and women >10.0%; 65 to 74 years of age, men >9.6% and women >9.6%; and 75 years of age or older, men >9.5% and women >9.6%) and a random blood sugar >200 mg/dL. Primary care physicians were consulted whenever there was doubt about the diagnosis.

The age at diagnosis of diabetes was defined as the age at the time of the diagnosis of diabetes as given by the participant or, if NIDDM was newly discovered, at the time of the examination. Current age was defined as the age at the time of the examination. The duration of diabetes was the time between diagnosis and the examination.

Statistics

The Statistical Analysis System (SAS, Cary, NC) was used for calculating prevalence proportions, means, chi-square statistics, and *t* tests.[16] Trends in proportions were tested for significance by the Mantel-Haenszel procedure.[17]

Results

Diabetic retinopathy was present in 36.8%, proliferative retinopathy in 1.8%, and macular edema in 3.0% of 435 people with known and newly discovered diabetes for whom gradable fundus photographs were available (Table 1). The highest frequency of retinopathy (68.4%), proliferative retinopathy (10.5%), and macular edema (10.5%) was found in the 19 people in whom diabetes was diagnosed before 30 years of age. Because this latter subgroup is small and previous studies have suggested that it is pri-

Table 2. The Relationship of Diabetic Retinopathy to Duration of Diabetes in the Beaver Dam Eye Study (1988–1990)

Duration (yrs)	Taking Insulin		Not Taking Insulin	
	No.	Retinopathy (%)	No.	Retinopathy (%)
<1	0	—	15	20.0
1-4	10	20.0	108	23.1
5-9	12	58.3	53	35.8
10-14	18	83.3	40	37.5
15-19	18	77.8	23	21.7
≥20	20	80.0	28	50.0
Test for trend	$P < 0.005$		$P < 0.02$	

marily composed of people with insulin-dependent diabetes, they have been removed from all further analyses.

We divided those who remained (n = 416) into 1 group with newly discovered NIDDM (n = 49) and 3 groups with previously diagnosed diabetes at 30 years of age or after: 1 in which people were using insulin (n = 79); 1 in which people were using oral hypoglycemic agents and/or diet (n = 271); and 1 in which people were using a combination of oral hypoglycemic agents and insulin (n = 17). The prevalence of retinopathy was lowest in people with newly discovered NIDDM (10.2%), highest in those whose diabetes was diagnosed at or after 30 years of age who were using insulin (69.6%), and intermediate in those who were using oral hypoglycemic agents and/or diet (29.9%) or oral hypoglycemic agents combined with insulin (35.3%) (Table 1). Proliferative retinopathy was present in 6.3% of subjects who were using insulin only and in 0.4% of subjects who were not using insulin. No one using insulin combined with oral hypoglycemic agents had proliferative retinopathy. No person in the newly diagnosed NIDDM group had signs of proliferative retinopathy; only one person had clinically significant macular edema.

The 5 people with retinopathy in the newly diagnosed NIDDM group were of a similar age (56.8 years versus 61.4 years, $P = 0.40$), had a higher systolic blood pressure (154.0 mmHg versus 136.2 mmHg, $P < 0.05$) and had similar diastolic blood pressure (76.8 mmHg versus 77.5 mmHg, $P = 0.90$) and body mass index (32.0 K/m^2 versus 33.5 K/m^2, $P = 0.60$) to the 44 people without retinopathy in the newly diagnosed NIDDM group.

The prevalence of any retinopathy increased with increasing duration of diabetes in both the previously diagnosed older onset group using insulin and the group not using insulin (Table 2). There was a significantly lower frequency of any self-reported visits to an ophthalmologist within 2 years of the examination by those with newly discovered NIDDM (24.5%) compared with those with previously diagnosed NIDDM (52.2%, $P < 0.001$).

Discussion

The Beaver Dam Eye Study, a large population-based study over a wide age range, provided an opportunity to evaluate the prevalence and severity of diabetic retinopathy in subjects discovered during the examination to have NIDDM. Although signs of diabetic retinopathy were present in approximately 10% of this group, none had proliferative retinopathy and only 2% had signs of clinically significant macular edema. These frequencies are similar to those reported in the Bedford Survey,[3] in which 6.9% of people with newly discovered NIDDM had diabetic retinopathy. Very low frequencies of retinopathy have been reported by others.[5,6] In the Rancho Bernardo population,[6] the frequency of retinopathy was only 0.6%, as detected by grading of fundus photographs in people with newly diagnosed NIDDM, defined by the presence of fasting hyperglycemia of ≥140 mg/dl and/or a 2-hour postchallenge blood glucose of ≥200 mg/dl. Similarly, Lundbaek,[5] using direct ophthalmoscopy, found that less than 1% of the Danish population studied had signs of diabetic retinopathy. However, higher frequencies of retinopathy have been described in other studies. Klein et al[4] reported retinopathy to be present in 21% of a group of 62 people 30 years of age or older within 2 years of diagnosis of NIDDM. In Beaver Dam, 20% of those with NIDDM had signs of retinopathy within 1 year of diagnosis. In the United Kingdom Prospective Study of Diabetes, Kohner et al found 23.8% of participants had diabetic retinopathy within 6 months of diagnosis of NIDDM (Kohner et al, personal communication).

Differences in reported prevalences of retinopathy in people with newly discovered NIDDM may be due to variations in the time between onset and detection of diabetes. This may be a result of socioeconomic factors, which determine the access to and availability of medical care, the health care seeking behavior of the specific group studied, as well as variations in the definitions used to define the presence of diabetes. Because the prevalence of diabetic retinopathy increases with increasing duration of hyperglycemia, retinopathy is more likely to be found in eyes of patients who have a longer interval between the onset of diabetes and its discovery.[4] Lower frequencies of retinopathy would be expected in asymptomatic people who are discovered to have diabetes by testing during population-based studies. These people are probably closer to the time of "onset" of their diabetes than symptomatic patients who are discovered to have NIDDM by their physicians. Defining the presence of diabetes by elevated blood sugars found during glucose tolerance testing may lead to a relatively earlier detection of milder diabetes compared with defining the presence of diabetes by elevated glycosylated hemoglobin in association with elevated random blood sugar as was used in the Beaver Dam Eye Study. This may, in part, explain the lower frequency of retinopathy found in the Rancho Bernardo population, with newly discovered diabetes compared with the frequency reported in the Beaver Dam population.[6]

Table 3. Comparison of Prevalence and Severity of Retinopathy in the Beaver Dam Eye Study (BDES) and in the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR)

	BDES Participants with Known Diabetes		WESDR Participants 43–86 Years of Age with Known Diabetes	
Worse Eye Retinopathy*	*No.*	*Percent*	*Estimated No.†*	*Percent*
10	231	59.8	2677.76	50.9
21	55	14.2	906.82	17.2
30	43	11.1	584.25	11.1
40	46	11.9	676.80	12.9
50	3	0.8	36.63	0.7
60+	8	2.1	382.30	7.3

* See Methods section for definition of retinopathy levels.
† The results are weighted to produce estimates for the entire population of diabetic persons. See Klein et al[4] for details of the weighting procedure.

Another cause of the differences in prevalence of diabetic retinopathy among people with newly discovered NIDDM is the dissimilarity in the sensitivity of techniques commonly used to detect retinopathy.[18–21] Ophthalmoscopy through an undilated pupil is less sensitive than grading of color stereoscopic fundus photographs of seven fields by experienced graders for the detection of retinopathy. The detection of diabetic retinopathy by grading only three standard fields, as done in the Beaver Dam Eye Study, is only slightly less sensitive than grading seven standard fields, as was done in other large epidemiologic studies such as the Wisconsin Epidemiologic Study of Diabetic Retinopathy.[22] Moss et al[21] found the sensitivity rate for any retinopathy was 92%, the rate for proliferative retinopathy was 86%, and the rate for proliferative retinopathy with Diabetic Retinopathy Study high-risk characteristics for severe visual loss was 87% when comparing findings from gradings of three standard fields with those derived from gradings of seven standard fields.

Furthermore, variations in the frequency of systemic conditions among people with diabetes (such as atherosclerotic carotid disease and hypertension) may result in the appearance of retinal microaneurysms and blot hemorrhages.[23] Although not a factor in Beaver Dam, the presence of acquired immunodeficiency syndrome could also result in an overestimate of "retinopathy."[24,25] Higher frequencies of these conditions may increase the rates of reported retinopathy in people with newly diagnosed diabetes.

These data suggest that asymptomatic people discovered to have NIDDM while participating in an epidemiologic study are probably not in need of immediate ophthalmoscopic examination to detect retinopathy at the time of discovery of NIDDM because it is unusual to discover either proliferative retinopathy or macular edema at that time. However, this is an opportunity to educate them about the future risk of visual loss due to diabetes and the importance of regular ophthalmologic care.[1] This has been suggested as being cost-effective for patients with newly discovered insulin-dependent diabetes mellitus,[26] although no data exist for people discovered to have NIDDM during screening or epidemiologic studies. Patients diagnosed with NIDDM by their physicians (not in a screening setting) may be at higher risk of having more severe retinopathy present at the time of their diagnosis.[4] They would be more likely to benefit from an immediate ophthalmologic examination.

The frequency of retinopathy increased with increasing duration of diabetes and was consistently higher in people using insulin compared with those not using insulin. These findings are consistent with previous studies and suggest the need for routine ophthalmologic care, especially for people with longer durations of diabetes.[4] In Beaver Dam, 48% of the people with known diabetes were not seen by an ophthalmologist within 2 years of their participation in the study. This is similar to findings from other population-based studies.[27,28]

There are no longitudinal national population-based estimates of the prevalences of retinopathy as determined by grading fundus photographs in the United States. Indirect evidence about temporal changes in the prevalence and severity of retinopathy obtained from blindness registries in England have been inconsistent.[29,30] The frequency and severity of retinopathy in the 395 people with known diabetes in the Beaver Dam Eye Study population between 1988 and 1990 was comparable with estimates from the similarly aged population participating in the Wisconsin Epidemiological Study of Diabetic Retinopathy[3,22] (also evaluated by a masked grading of fundus photographs using the modified Airlie House Classification[12–14]) studied from 1980 to 1982, suggesting little change over the 8-year interval between the studies (Table 3).

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